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Channel Catfish Virus: a New Herpesvirus of Ictalurid Fish

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Channel catfish virus was studied in ictalurid fish cell culture, the only system of fish, amphibian, avian, and mammalian cells found to be susceptible. Channel catfish virus infection resulted in intranuclear inclusions and extensive syncytium formation. Replication occurred from 10 to 33 C, but not higher. Best growth was from 25 to 33 C, and the amount of virus released nearly equalled the amount which remained cell-associated. The virus was labile to lipid solvents, and indirect determinations with labeled precursors and a metabolic inhibitor showed evidence of deoxyribonucleic acid. Electron microscopy showed progeny virus, about 100 nm in diameter, in various stages of development in cell nuclei by 4 hr. Present also were nuclear masses of exceptionally electron-dense lamellar material, with a unit dimension of 10 to 15 nm. Virus was enveloped at the nuclear membrane and in cytoplasmic vacuoles, resulting in virions having a diameter of 175 to 200 nm. Negative staining demonstrated icosehedral symmetry and 162 capsomeres. Our data indicate that channel catfish virus is a herpesvirus.

The most numerous of the poikilothermic vertebrate viruses are those found in teleost fishes, and there are representatives in several of the currently recognized major groups of animal viruses (1). The fish viruses have not been extensively studied, but they warrant greater attention because of the temperature range through which they replicate, because of their relationship to other animal viruses, and especially because of their exclusively aquatic ecology.

A virus was isolated from four of five epizootics of an acute hemorrhagic disease of high mortality in populations of young channel catfish (*Ictalurus punctatus*) by Fijan, Wellborn, and Naftel (4). The etiological agent was termed channel catfish virus (CCV). It passed 0.22-μm pore-size filters and could be propagated only in cells of ictalurid fishes.

Because of the potential economic importance of this virus as a fish pathogen, particularly in hatchery situations, and in the interest of extending our knowledge of the comparative virology of the lower vertebrates, we have studied the biological and morphological aspects of the growth of CCV in a cell culture system.

The virus proved to be quite similar biologically and morphologically to herpesviruses from homothermic vertebrates although there are differences in temperature requirements and in certain morphological aspects of development.

MATERIALS AND METHODS

Virus and cells. The original isolate of CCV, designated strain Auburn, was provided by N. N. Fijan of Auburn University for our work. CCV was cloned and grown in BB cells, a continuous line of mixed epithelial fibroblast-like morphology, initiated from caudal trunk tissues of the brown bullhead (*Ictalurus nebulosus*), a teleost fish. A starter culture of BB cells was provided by the originators, C. P. Cerini and R. G. Malsberger, Lehigh University, Bethlehem, Pa.

Cells were grown at 22 to 30 C in Eagle minimal essential medium with 10% fetal bovine serum (MEM-10). The same medium with 2% fetal bovine serum (MEM-2) was used for maintenance during virus growth or assay procedures.

Plaque assays were done in confluent monolayers of BB cells in 60-mm plastic dishes (Falcon Plastics, Los Angeles, Calif.) by using a modification of the method described by Moss and Gravell (9). A 0.1-ml amount of serial 10-fold dilutions of virus was allowed to adsorb for 1 hr at 22 to 26 C, after which the monolayers were overlaid with 2.0 ml of MEM-2 containing 1% Agarose (Sea Kem Corp.). After solidification, the gel was covered with 4 ml of MEM-2. Incubation was continued at 30 C for 40 $\pm$ 4 hr, and plaques were made visible by fixing the cell sheet with formalin, decanting the gel, and staining with 1% crystal violet in 95% ethanol or in water.

For virus growth curves, confluent cell sheets in 60-mm petri dishes were inoculated with 0.1 ml of virus suspension at multiplicities of 25 to 141 plaque-forming units (PFU) per cell. After 1 hr of absorption, the cultures were washed three times with 5 ml of Hanks salt solution containing 0.5% lactalbumin hydrolysate (HLAH), 100 international units of penicillin, and 100 μg of streptomycin per ml. After washing, 5 ml of MEM-2 was added. At 25 C and above, the cultures were incubated in 5% CO_2 and air. Below 25 C, the MEM-2 was buffered with 6.7 mM $NaHCO_3$ and 14 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Calbiochem, Los Angeles, Calif.) at *p*H 7.8 and incubated in humidified chambers in air. Samples were collected at selected intervals, and the medium was removed and stored at 4 C until assayed for released virus. The cells were scraped into 5 ml of MEM-2, disrupted by 1 min of sonic treatment (Raytheon, 10 kc at maximum output), and similarly stored until assayed for cell-associated virus. All assays were made on duplicate plates.

Light microscopy. Cover slip cultures were inoculated with virus and incubated at 30 C. At regular intervals, sample cultures were drained, washed three times with HLAH, fixed with absolute methanol, and stained with May-Grunwald-Giemsa stain (7).

Electron microscopy. Monolayers grown at 30 C were washed once with phosphate-buffered saline, scraped from the surface of the dish, and sedimented at 200 to 500 $\times$ *g* for 6 to 10 min. Cell pellets were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate and postfixed in 1% osmium tetroxide. After dehydration in graded alcohols, the cells were imbedded in Epon 812. Sections were stained with uranyl acetate (15) and lead citrate (13) and examined in a Siemens Elmiskop I electron microscope at 80 kv accelerating voltage.

For negative staining, the virus particles were partially purified by differential centrifugation, resuspended in phosphate-buffered saline or 0.1 M ammonium acetate, and stained with 2% ammonium molybdate adjusted to *p*H 6.8.

RESULTS

Virus growth and cell susceptibility. CCV grew in BB cells at temperatures of from 10 to 33 C, but the best replication occurred at 25 C and above. Growth did not occur at 37 C. Diluted preparations produced well-defined plaques (Fig. 1) under Agarose overlays. A representative growth curve at 30 C is shown in Fig. 2. The logarithmic growth phase began at 4 to 5 hr after infection, and the plateau was reached by 10 to 12 hr. There was always slightly more cell-associated virus than released virus in the samples. Growth curves done at 25 to 33 C were essentially the same as at 30 C. At lower temperatures (10 to 25 C), the slope of the curve was unchanged, but the length of the eclipse period and the titer attained were inversely proportional to the temperature.

CCV did not produce cytopathic effects or infectious virus in cell cultures from 14 different animal species. These included rainbow trout gonad (RTG-2), fathead minnow (FHM), and bluegill fry (BF 2), all established lines from teleost fishes. Amphibian cells were bullfrog tongue (FT) and 3AKRP, the latter an undescribed line developed by Maneth Gravell from adult *Rana pipiens*. Avian cells were primary chick

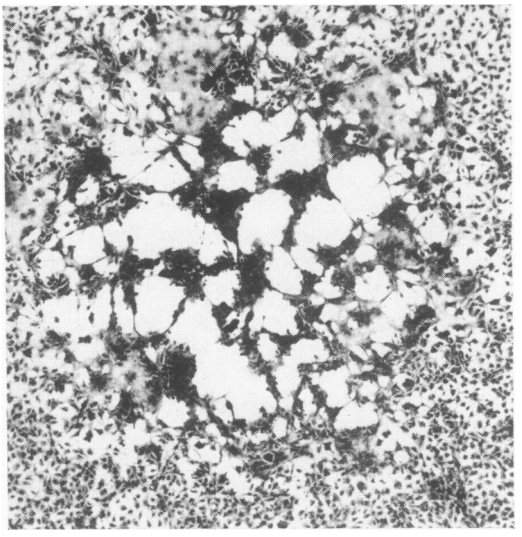

FIG. 1. *CCV plaque in BB cells.*

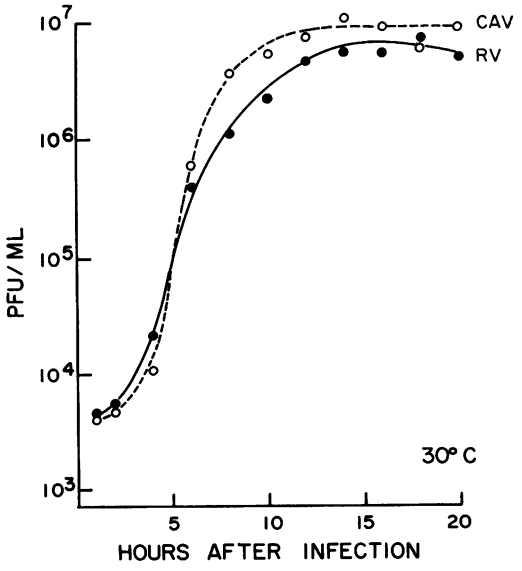

FIG. 2. *Representative growth curve of CCV in BB cells at 30 C; CAV is cell-associated virus; RV is released virus.*

embryo. Cells from mammals consisted of primary kidney cultures from rabbit, rhesus monkey, African green monkey, and human embryo, and the established lines HEp-2, WI 38, HeLa, and BHK-21.

Sequence of cellular changes. Early in infection, cell nuclei became basophilic, and by the end of the 2nd hr there was margination of chromatin and beginning syncytium formation. Intranuclear inclusions were evident in some cells by the 3rd hr, and by the 4th hr they were in most nuclei (Fig. 3). The inclusions differed somewhat from the classical Cowdry type A, in that they were quite granular and irregular in shape. Recruitment of cells into syncytia continued, and at the 6th hr there was beginning disintegration of nuclei, and basophilic condensations—presumably nuclear origin—appeared in the cytoplasm. By the 8th hr, syncytia were contracting and nuclear disintegration was common. By the 10th hr, the cytoplasmic portions of syncytia were undergoing fragmentation or lytic disintegration. From the 12th to the 14th hr nuclear and cytoplasmic disintegration continued.

The effect of virus on BB cells was related to the multiplicity of infection. At multiplicities of less than 1 PFU/cell, syncytium formation was enhanced, and many hundreds of nuclei collected beneath a common membrane (Fig. 4). High multiplicities (50 to 100 PFU/cell) resulted in very early cellular pycnosis, and syncytia typically consisted of relatively few cells.

As BB cells had not been examined by electron microscopy, a survey of uninfected cells was done to provide a baseline for the study of infected cells. These cells (Fig. 5) had irregularly shaped nuclei with prominent nucleoli. Moderate numbers of densely staining mitochondria were present in the cell cytoplasm along with an extensive system of endoplasmic reticulum. The plasma membrane was convoluted to a degree which made it difficult to define cell boundaries. Very prominent desmosomes (arrows and insert, Fig. 5) were commonly seen connecting two apposing plasma membranes.

Virus development. A sequential study of virus development at 30 C was done by electron microscopy with samples taken during the eclipse, logarithmic growth, and plateau phases. No attempt was made to study the early (attachment and penetration) events.

At 1 and 2 hr after infection, there was no evidence of progeny or input virus. At 3 hr, no virus structures were seen, but the chromatin was marginated in the nuclei of occasional cells. By 4 hr, virus synthesis was evident in most of the cells (Fig. 6). Scattered randomly throughout the nucleus were virus particles in all stages of development. Virus particles were often seen associated with structures of widely varying size made up of granules 15 to 20 nm in diameter (Fig. 6, arrow A; Fig. 7). Extremely electron-dense lamellar structures first appeared at this time (Fig. 6, arrow B). The nuclear membrane was indistinct in some areas (Fig. 6, arrow C; Fig. 8, arrow B), and the nuclear and cytoplasmic compartments appeared to be continuous with each other. Many unenveloped particles were seen in the cytoplasm (Fig. 6). These usually contained dense nucleoids and were concentrated in certain areas of the cytoplasm, rather than being distributed randomly.

Three distinct types of nuclear particles were seen. These were single-membraned particles with electron-lucent centers (Fig. 7, arrow A), double-membraned particles (Fig. 7, arrow B), and single-membraned particles with electron-dense cores (Fig. 7, arrow C). These cores were either

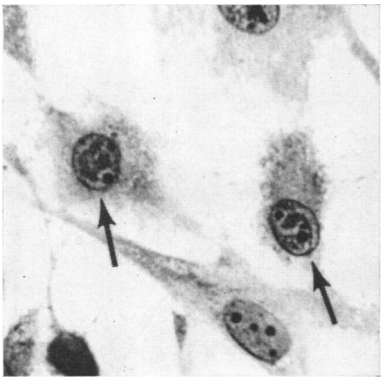

FIG. 3. *CCV-infected BB cells showing intranuclear inclusions (arrows).*

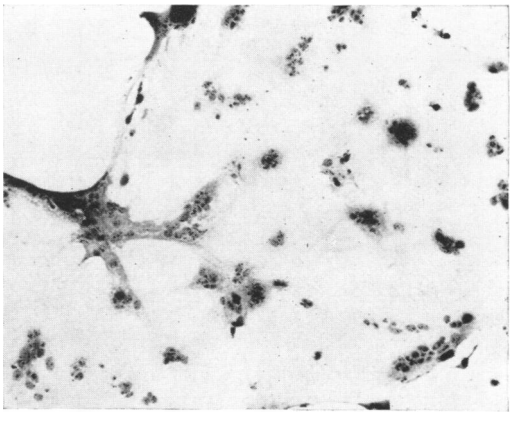

FIG. 4. *Large syncytium resulting from infection of BB cells by CCV.*

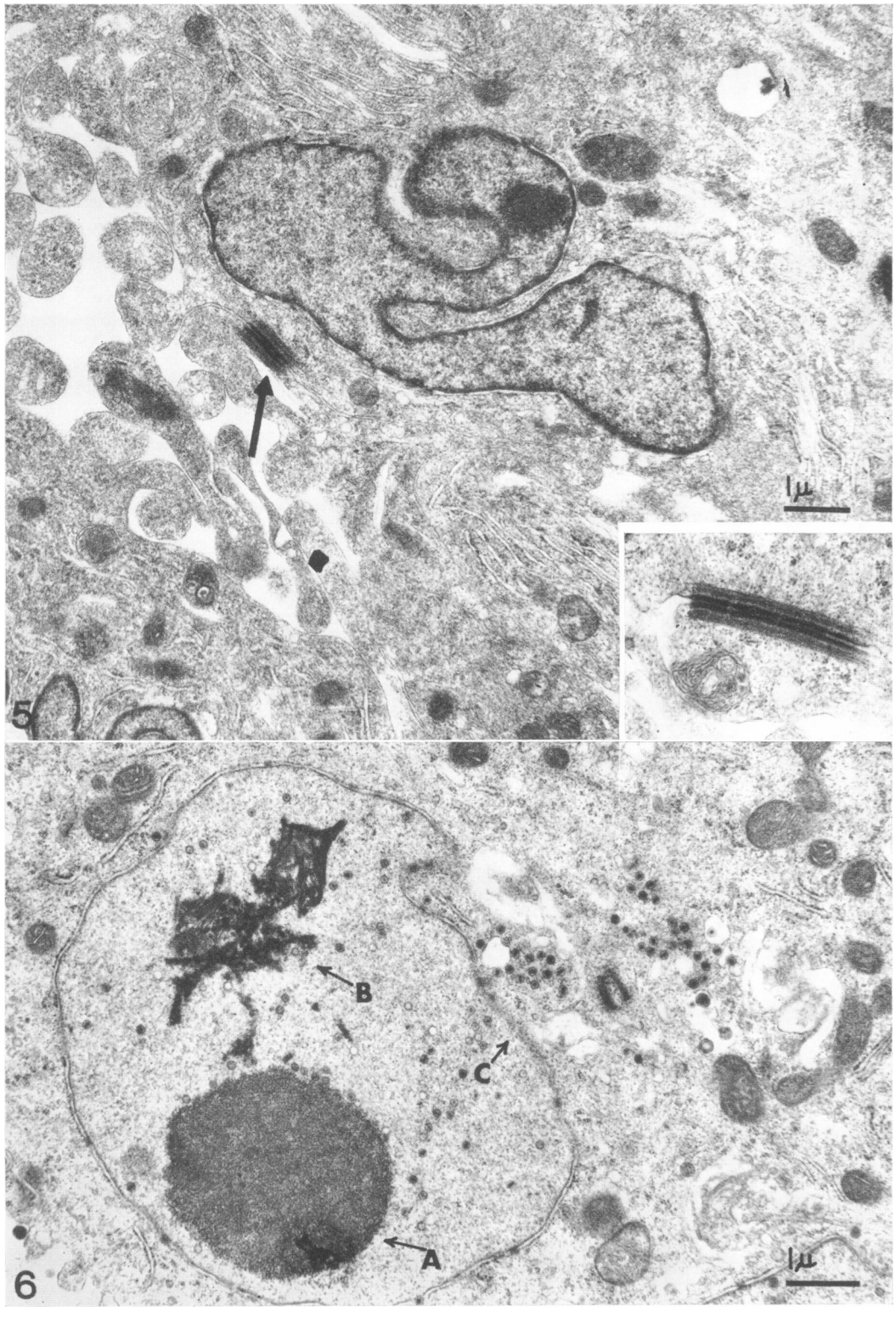

FIG. 5. *Uninfected BB cells with convoluted nucleus, extensive rough endoplasmic reticulum, and desmosome (arrow). Insert shows detail of desmosome. Magnification, ×10,000.*

FIG. 6. *BB cell 5 hr after infection with CCV. Virus particles in all stages of development in nucleus. Granular body (A) with associated virus particles; lamellar structure (B) and indistinct section of nuclear membrane (C) are evident. Numerous unenveloped viral particles are present in the cytoplasm. Magnification, ×11,000.*

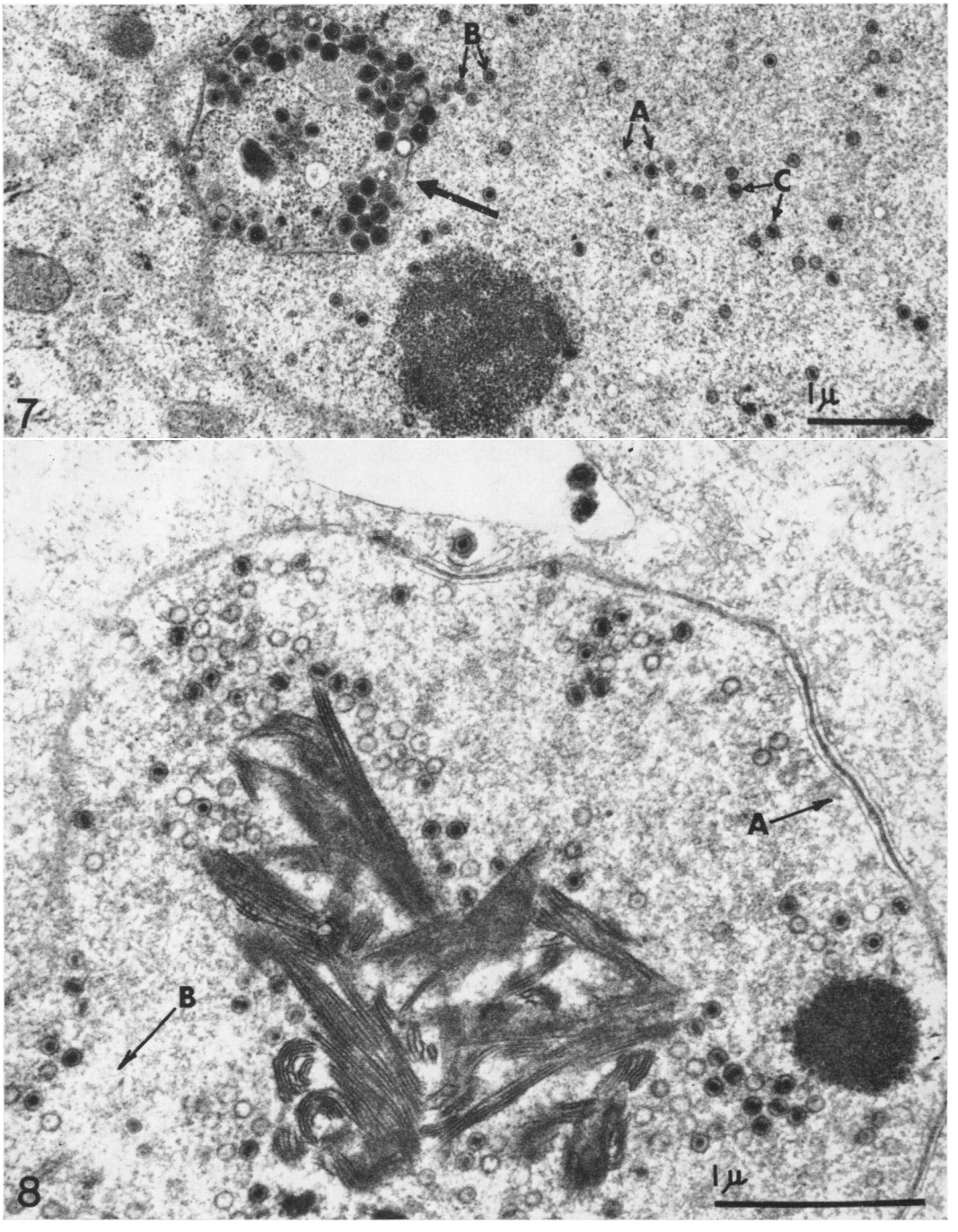

FIG. 7. *BB cell 10 hr after infection with CCV. Vacuole (large arrow) is present at nuclear periphery containing primarily enveloped particles. Three types of developing virus particles are seen: A, single-membraned particles with electron-lucent centers; B, double-membraned particles with electron-lucent centers; C, single-membraned particles with bar-shaped electron-dense cores. Magnification, ×18,000.*

FIG. 8. *BB cell 10 hr after infection with CCV. There is extensive reduplication of the nuclear membrane (A) and in some areas it appears disrupted (B). A dense lamellar structure occupies a large portion of the nucleus. Magnification, ×30,000.*

rounded up or bar-shaped. Unenveloped virus particles were from 95 to 105 nm in diameter. The cores were from 40 to 50 nm in diameter. Although aggregates of virus particles were common, crystalline arrays were not seen. Vacuolar structures (Fig. 7, large arrow) were often present at the nuclear periphery. These contained enveloped particles primarily, although a few unenveloped particles were also present.

Reduplication of the nuclear membrane (Fig. 8, arrow A; Fig. 9) was commonly seen in infected cells. This consisted of an electron-dense center lamella of irregular width ranging from 30 to 40 nm, with thinner lamellae on either side.

The lamellar structures (Fig. 6, 8, 9), which first appeared at 4 hr after infection, consisted of very dense lamellae 10 to 15 nm wide in parallel arrays. The lamellae were separated from each other by irregular spaces, and the number of lamellae in separate arrays was not consistent, varying from 2 to 8 or 10. Late in infection (16 to 24 hr), the arrays filled large areas of the nucleus (Fig. 9).

At 6 to 8 hr after infection, portions of the nuclear membrane of some cells were indistinct, and by 16 to 24 hr many cells had large segments of the nuclear membrane missing (Fig. 9). This resulted in the release of large numbers of unenveloped particles into the vacuolated cytoplasm. Virus particles were enveloped by budding into cytoplasmic vacuoles (arrows, Fig. 10). Virus particles could also be found budding from the inner lamella of the nuclear membrane into the perinuclear cisterna (insert, Fig. 10).

Virus particles outside the cell (Fig. 11) were usually enveloped and contained cores. The enveloped particles were approximately 175 to 200 nm in diameter, and electron-dense amorphous material was often present between the nucleocapsid and the envelope.

Examination of negative stains revealed that the nucleocapsid was made up of hollow capsomeres and was icosahedral (Fig. 12). In preparations which had particles oriented so that an edge of a triangular facet could be clearly discerned, five capsomeres could be seen along this edge. This would indicate that the particle is composed of 162 capsomeres.

DISCUSSION

The evidence presented here indicates that this viral pathogen of fish is a herpesvirus. The findings which bear on this conclusion are: the presence of intranuclear inclusions and syncytia, as shown by light microscopy; assembly of the virus in the nucleus and acquistion of an envelope at the nuclear membrane and in the cytoplasm, as shown by thin-section electron microscopy; and size (100 nm) and number of capsomeres (162), as demonstrated by negative staining.

Other evidence (Wolf, *unpublished data*) has been obtained which serves to link this virus to the herpesvirus group. Virus infectivity was completely inactivated by treatment at 4 C with 20% ether for 24 hr and by treatment with 5% chloroform for 5 min at room temperature. In addition, pulse-labeling experiments demonstrated that there was a five to sixfold increase in incorporation of tritiated thymidine by CCV-infected cells at 4 hr after infection and a comparable decrease in incorporation of tritiated uridine at 6 hr after infection. These results would seem to indicate that the thymidine precursor is being incorporated into viral deoxyribonucleic acid. Considered alone, the data are open to a second interpretation—that CCV stimulates deoxyribonucleic acid synthesis as do papovaviruses (1, 15). CCV replication, however, is completely inhibited by 10^{-4} M iododeoxyuridine.

The pattern of CCV development exhibits many features in common with other herpesviruses and has the characteristics of a group A herpesvirus (6). That is, there is extensive release of infectious virus into the supernatant fluid of infected cultures, and by 4 hr after infection, there is an increase in progeny virus.

Granular structures similar to those shown here (Fig. 6–8) have been reported in other herpesvirus-infected cells by Morgan et al. (8) and others. Although virus particles in all stages of development were seen in close proximity to these structures, there were many more such particles scattered randomly throughout the nucleoplasm. Thus, the question of whether these structures play a role in virus synthesis and assembly is still open.

The lamellar structures which appear in the nucleoplasm of BB cells infected with CCV have no exact counterpart in other herpesvirus infections. Filamentous forms have been described in nuclei infected with infectious larngyotracheitis virus (16), equine rhinopneumonitis virus (11), mouse cytomegalovirus (12), and herpes simplex virus (2, 8, 10). We have observed hollow filaments in type 2 herpes simplex infections of BHK, HeLa, and HEp-2 cells, but not in type 1 (Darlington, *unpublished data*). Such filaments have also been reported in Lucké tumor cells carrying the herpes-type virus (5, 14). In all of these reports, the structures have been described as tubular filaments, and in some instances they have been interpreted as being aberrant virus particles. The structures occurring in CCV-infected cells appeared quite different in most respects. They were very electron dense

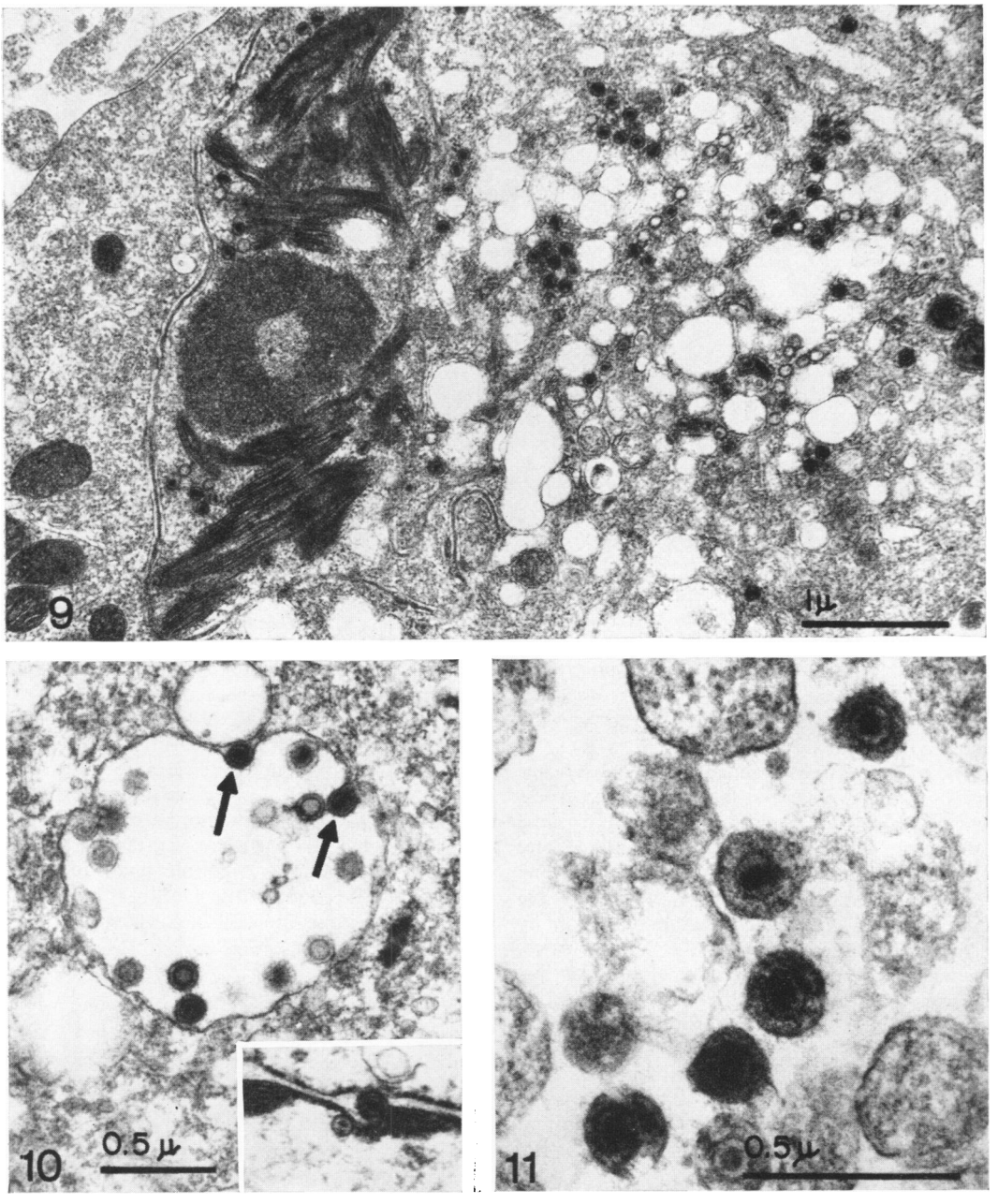

FIG. 9. *BB cell 24 hr after infection with CCV. Dense lamellar structure is very extensive; reduplicated nuclear membrane is disrupted along the right-hand side. Magnification, ×20,000.*

FIG. 10. *BB cell 24 hr after infection with CCV. Cytoplasmic vacuole containing enveloped virus. Virus particles (arrows) are in the process of budding into the vacuole. Insert shows a virus particle acquiring an envelope at the inner lamellae of the nuclear membrane. Magnification, ×32,000.*

FIG. 11. *Extracellular CCV particles. Particles are enveloped; electron-dense material is present within the envelope. Magnification, ×60,000.*

and occurred as stacks of lamellae, rather than as tubular structures. In addition, they were much more prevalent than those previously reported. By 15 hr or later, every nucleus in the infected cultures contained the lamellae, and they eventually occupied most of the nucleus. They may be the result of aberrant viral assembly, although this seems unlikely in view of the

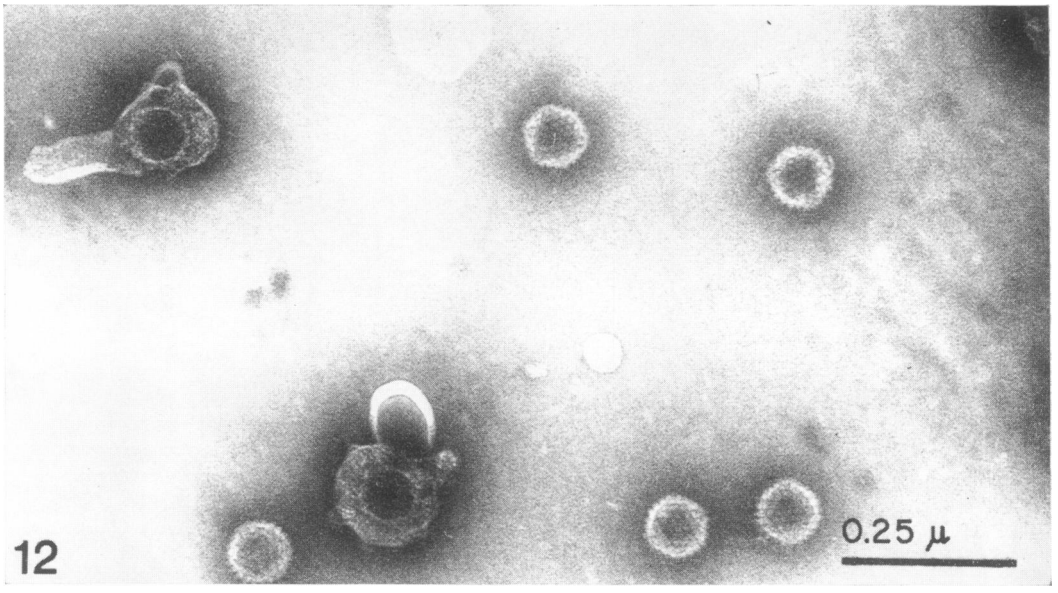

FIG. 12. *Negatively stained CCV particles. Magnification, ×90,000.*

profusion in which they occur. Alternatively, they may be a cellular response to insult by the virus. As they were seen only in infected cells, it appears inescapable that they, in some way, resulted from virus infection.

The method of envelopment of the nucleocapsid is similar to that seen in many other herpesvirus infections (3), although there are some differences. Envelopment occurs at the inner lamellae of the nuclear envelope and by budding into nuclear vacuoles. There is also extensive reduplication of the nuclear membrane. In many instances, in contrast to most other herpesviruses, the nuclear membrane appears ruptured or disintegrated and, as a result, many unenveloped particles appear in the cytoplasm. These acquire envelopes by budding into cytoplasmic vacuoles which occur in large numbers in infected cells. This process is in accord with the concept (3) that the envelopment process is nonspecific and occurs at any membrane the unenveloped particle encounters.

The origin of this virus of teleost fishes is of considerable interest. One possibility is that it evolved independently in its present host, arriving at a morphological state similar to that of the herpesviruses of warm-blooded vertebrates. Fish arose in the Devonian era before other vertebrates, and ancestral catfish originated in North America in mid-Eocene approximately 50,000 years ago. The fact that CCV is highly virulent for young channel catfish must be considered in the light of its known occurrence. At present it is restricted solely to young channel catfish propagated under crowded hatchery conditions. In that respect, CCV follows the pattern of epizootic mortality caused by two other viruses among hatchery-propagated salmonids in North America (18). The apparent virulence need not argue against maintenance of a successful host-parasite relationship, but may be simply a result of husbandry in which the virus is given the advantage of high numbers of susceptible hosts in comparatively close confinement. Until it is known to be otherwise, one must also consider CCV as being exotic in the channel catfish. Because only ictalurid fish cells are susceptible, it is likely that CCV originates in an ictalurid fish. The white catfish (*I. catus*) is only slightly susceptible and might well be a reservoir. The other, probably more remote, possibility is that a herpesvirus of warm-blooded animals has become adapted to growth in this poikilotherm and has lost its ability to grow at higher temperatures. If one can obtain temperature-sensitive mutants in the laboratory, there is no reason to think that nature cannot do the same. There is, of course, no information at hand bearing on these speculations. It would be of particular interest to compare the antigenic structure of this virus with that of the other herpesviruses, particularly the herpesvirus associated with the Lucké tumor because of the phylogenetic relatedness of their hosts.

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