American Fisheries Society Special Publication 18:23–37, 1988
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Epizootic Diseases of Oysters Associated with Viral Infections

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Abstract.—Virus infections have been associated with major diseases of oysters of the genus *Crassostrea*. These infections include gill necrosis virus and hemocytic infection virus diseases of the Portuguese oyster *C. angulata* and more recently the oyster velar virus disease affecting larval, hatchery-reared Pacific oysters *C. gigas*. This report presents histo- and cytopathological characteristics of these infections in which large icosahedral cytoplasmic deoxyriboviruses, similar to the Iridoviridae, are implicated. The gill necrosis virus causes, principally in the Portuguese oyster, an evolutive ulceration of the gills, including cellular hypertrophy and severe inflammation. Mortalities have been observed in the most serious cases. Hemocytic infection virus has caused mass mortalities of Portuguese oysters in Europe (1970–1974); it induces cytoplasmic lesions in the hemocytes and causes severe injuries to interstitial tissues. Similar infections were observed in 1977 in Pacific oysters. The oyster velar virus develops in the cytoplasm of epithelial cells of the velum and causes tissue lesions. It is the apparent cause of some hatchery mortalities.

Until recently, much of our knowledge of epizootic mortalities of molluscs associated with microbial disease agents has been restricted to bacterial, mycotic, and protozoan diseases, and little attention has been given to viral diseases. During the past decade, important virological studies have led to the discoveries of viral diseases in molluscs, particularly molluscs of economic importance.

Although a virus has been suspected as the etiologic agent of the epizootic Malpeque Bay disease of the eastern oyster *Crassostrea virginica* (Rosenfield 1969), the actual occurrence of a viral infection in molluscs was first demonstrated by Rungger et al. (1971) in the common octopus *Octopus vulgaris*. In the same year, Devauchelle and Vago (1971) noted the presence of viruslike particles, similar to reoviruses in the cuttlefish *Sepia officinalis*.

The first case of viral infection found in marine bivalve molluscs was described in the eastern oyster by Farley et al. (1972), who reported hexagonal, single-enveloped, herpeslike virus particles in tissues of eastern oysters subjected to elevated water temperatures. The viral particles were empty or possessed dense nucleoids, and the enveloped virions (200–250 nm) were observed in the cytoplasm of infected cells (Farley 1978). Subsequently, from electron microscope studies, viruslike particles were recognized in several species of oysters. Ribonucleic acid-like, enveloped viral particles, 100–110 nm, were noted by Farley (1976) in the epithelial cells of the digestive diverticula of *C. virginica*. This author also described virus particles (53 nm) associated with marked hypertrophy of gametogenic epithelial cells of *C. virginica* and similar lesions in tissues of *C. gigas* (Farley 1976). He later observed, in the hemocytes of the Olympia oyster *Ostrea lurida*, viruslike ribonucleic acid particles budding through the plasma membranes of the cells and enveloped virions (50 nm) and conforming to the morphological description of Togaviridae.

While these cases of viral infections, which are rare and of little pathological importance, were reported, other new viroses developing in epizootic form were observed during major disease outbreaks of oysters: gill necrosis virus disease and hemocytic infection virus disease (HIVD) associated with mass mortalities of the Portuguese oyster *C. angulata* (Comps and Duthoit 1976; Comps et al. 1976) and HIVD observed in the Pacific oyster *C. gigas* during summer mortalities in the Bay of Arcachon (France) in 1977 (Comps and Bonami 1977). The pathogenic agents of these diseases have properties resembling iridoviruses.

A third type of iridovirus, the oyster velar virus (OVV), has been found in the velar epithelial cells of hatchery-reared, Pacific oyster larvae, which were undergoing serious mortalities (Elston 1979).

Gill Necrosis Virus and Hemocytic Infection Virus Diseases

Both gill necrosis and hemocytic infection viruses (GNV, HIV) have been implicated in epizootic diseases of the Portuguese oyster and have very similar characteristics including size, shape,

and intracytoplasmic morphogenesis; however, they have different pathogenic effects.

Hosts

Although GNV infection was observed more commonly in the Portuguese oyster, a few cases of this infection have been observed in the diseased gills of Pacific oysters cultured in the region of Marennes–Oléron, France (Comps 1969; Comps 1970; Comps and Duthoit 1976).

Hemocytic infection virus caused serious infection and mass mortalities of Portuguese oysters in France from 1970 to 1973 (Comps et al. 1976). A similar type of virus infection has been reported in Pacific oysters in the Bay of Arcachon long after the disappearance of the Portuguese oyster (Comps and Bonami 1977).

Viruses

Morphology.—Mature particles (380 nm in diameter) scattered through the cytoplasm of infected cells have an icosahedral symmetry, with five-, three-, and twofold axes of rotational symmetry to their sections. The shells of the virions appear to consist of two trilaminar layers (Figure 1A, B). However, some sections show that the outer layer is composed of subunits, corresponding to an icosahedral lattice. A fringe of knobbed fibrils is attached to the subunits (Figure 1B).

The electron-opaque central core, 250 nm in diameter, is limited by a three-layered fringe of definite width and surrounded by a layer of dense material. Occasionally, sections of the core reveal an ordered stacking of the fine filaments, which may represent the nucleic acid arrangement. These features of the shell conform to the model established by Stolz (1973) for icosahedral cytoplasmic deoxyriboviruses (ICDV).

Morphogenesis.—The morphogenesis of these viruses takes place in the cytoplasm of oyster hemocytes or gill cells (Comps 1983). Several distinctive features occur during this process.

At the periphery of the virogenic stroma, a trilaminar element develops similar to the inner membrane which constitutes the inner component of the shell (Figure 2A). In juxtaposition to the unit membrane, in association with the development of the shell, a layer of poorly defined subunits appears; the subunits induce a sharp outline to the incomplete particles due to the increased density of the virogenic granular material (Figure 2B). Then the immature particle becomes detached from the viroplasmic matrix (Figures 1D, 2C); a dense nucleoid with two layers is contained within the shell of the developing particle. The external layer is formed of subunits, while the inner layer corresponds to a unit membrane.

The maturation stages of the particles are visible during the pathogenesis of gill disease. They are observed during the development of the electron-dense nucleoid, which retracts from the inner surface of the shell (Figure 1D).

Nucleic acid.—The Portuguese oyster viruses were not isolated from the oysters during the course of the disease, nor were they characterized chemically. The presence of a deoxyribonucleic acid (DNA) virus was demonstrated by histochemical techniques. Fluorescent microscopy of histologic sections stained with acridine orange at pH 3.8 revealed large greenish-yellow areas that fluoresced in the cytoplasm of cells infected by GNV. The intensification of the emission of fluorescence indicated DNA concentrations compatible with the replication of the observed virus. Similar observations that were made with the fluorochrome 4′,6-diamidino-2-phenylindole (DAPI) confirmed the virus of gill disease to be a DNA virus (Comps and Masso 1978). The same techniques also confirmed HIV as a DNA virus.

Pathologic Lesions

Gill disease (GNV infection).—Portuguese oysters infected with GNV exhibit various stages of ulcerations of the gill and labial palps (Comps 1970). Three stages of lesion development have been described according to the extent of tissue damage. The first detectable gross lesions are small perforations in the center of yellowish discolored zones of tissue. Further development and extension of the lesions result in larger and deeper ulcerations with jagged edges (Figures 3, 4).

Histologically, the response to the infection is characterized by tissue necrosis and massive hemocytic cellular infiltration around the lesions; the infiltration induces marked visible changes in the structural organization of the gills. The gill filaments, the interfilamentar junctions, and the interlamellar septae can be affected (Figure 5). Marked inflammatory reactions are observed in the connective tissue of ulcerated labial palps (Figure 5D).

The most distinctive cellular lesion associated with the disease is the occurrence of giant polymorphic cells, which may be up to 30 μm in size. The nucleus of the cell is hypertrophied and usually oval; its maximum width is 10 μm and its length is 15 μm. The diameter of the nucleolus is between 2 and 4 μm. The cytoplasm contains

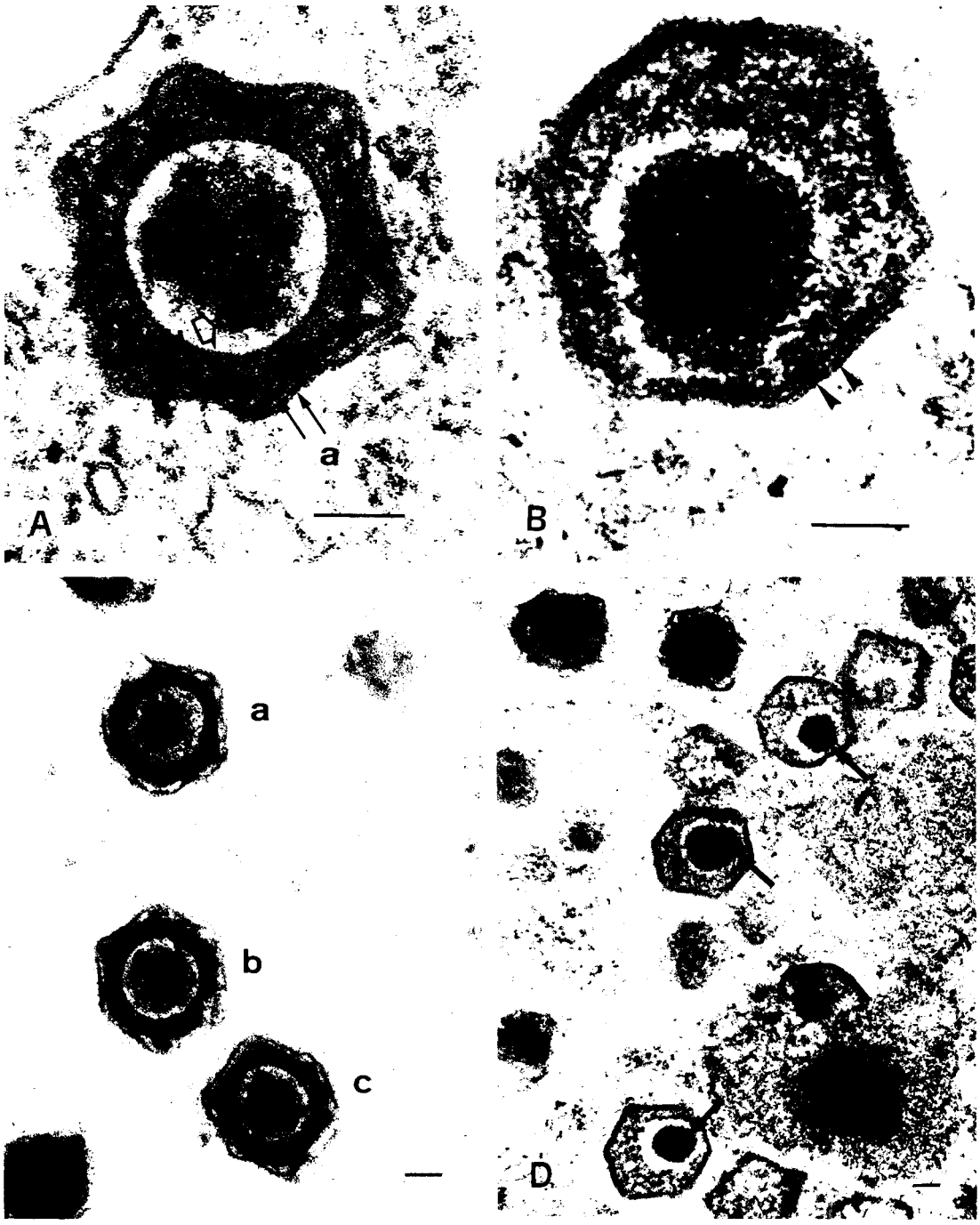

FIGURE 1.—Morphology of the hemocytic infection virus (HIV) and the gill necrosis virus (GNV). **A.** Characteristic HIV mature particle. a = trilaminar elements forming the shell, b = central core, c = three-layered fringe, d = filament, possibly representing nucleic acid arrangement. **B.** Section of GNV immature particle showing the structural subunits of the outer element of the shell (arrows). **C.** HIV particles exhibiting different axes of rotational symmetry. a = fivefold axes, b = threefold axes, c = twofold axes. **D.** Stages of the development of electron-dense nucleoids (arrows) into immature GNV particles. Electron micrographs; bars = 100 nm.

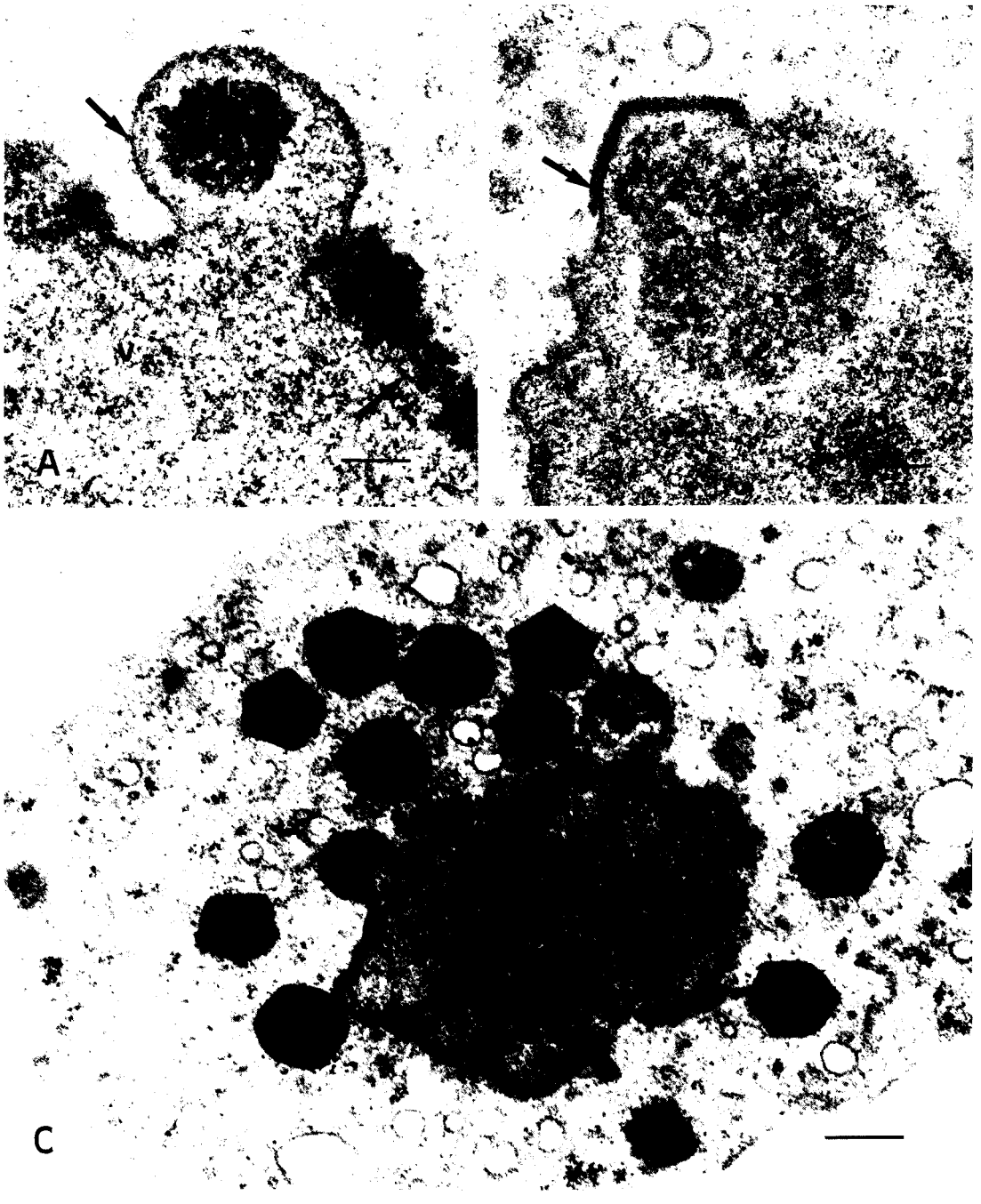

FIGURE 2.—Electron micrographs of the sequence of morphogenetic events involved in the formation of the virions. **A.** Development on the periphery of the virogenic stroma (v) of a trilaminar structure (arrows) typical of a unit membrane. Bar = 100 nm. **B.** Addition of outer layer of subunits (arrow). Bar = 100 nm. **C.** Separating immature hemocytic infection virus particles. Bar = 300 nm.

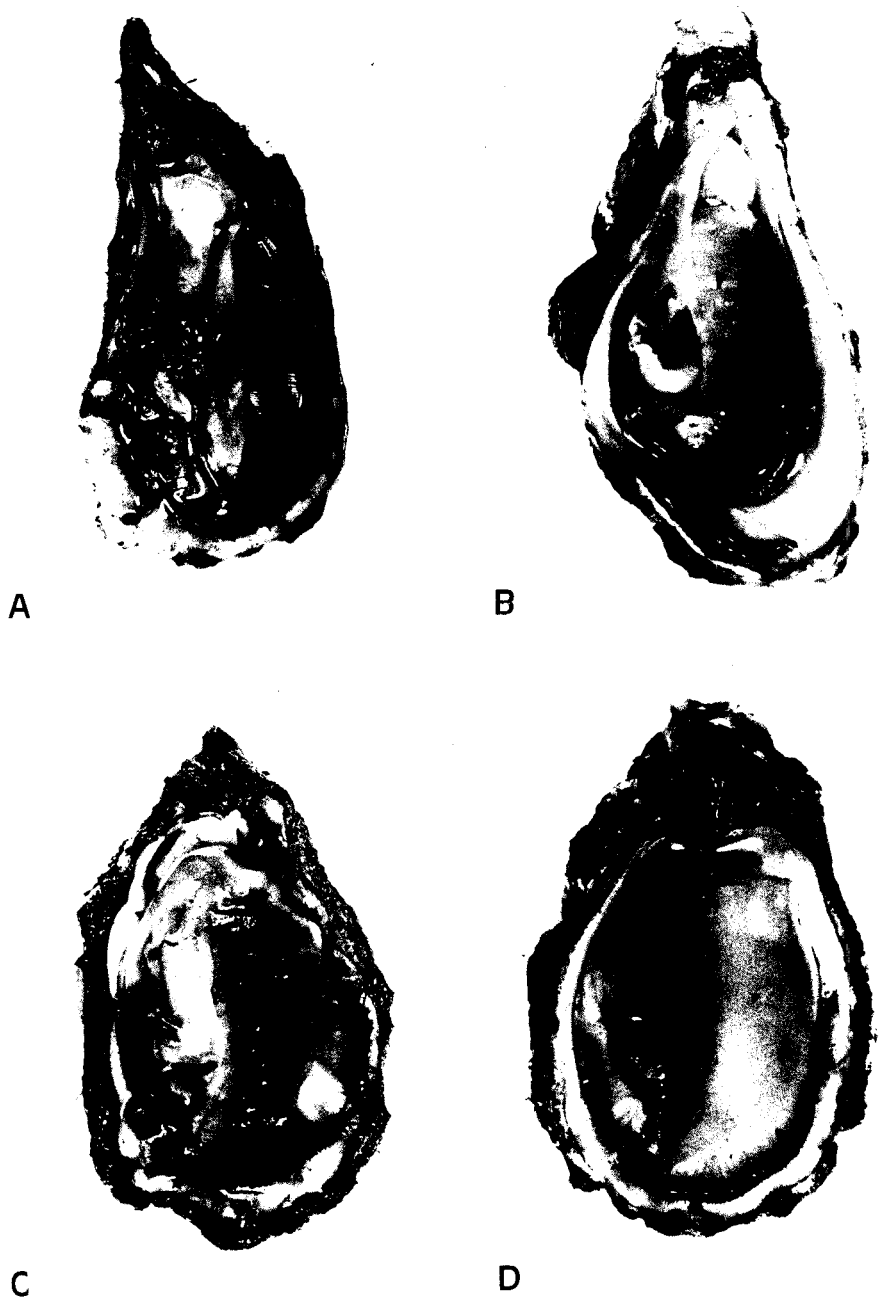

FIGURE 3.—Evolutive stages of the gill necrosis virus disease in the Portuguese oyster. **A.** Healthy oyster. **B.** Microperforations associated with inflammation on the gills (arrows). **C.** Severe ulceration of the gills exhibiting deeply jagged edges (arrow). **D.** Prelethal stage: the gills are nearly destroyed, and the oyster is very thin.

FIGURE 4.—Detail of a typical lesion of the gill necrosis virus disease. The necrosis of the tissues (arrow) induces major alterations in gill morphology. Bar = 1 mm.

large, lightly refringent fuchsinophilic granules, 3 to 5 μm in diameter, that are frequently associated with small basophilic granules and vacuoles (Figure 6). In some giant cells, generally found at the edge of necrotic lesions, a voluminous basophilic inclusion (5–15 μm) occupies the greatest part of the cytoplasm in which finer basophilic grains (0.4–0.5 μm) are also present (Figure 7). The presence of DNA in the inclusion body is indicated by a positive reaction when the inclusion body is stained by the Feulgen method, and the reaction is confirmed by the acridine orange and DAPI reactions, yielding intense yellow-green and blue staining, respectively. In some cases, the same cytochemical properties can be observed in the fine granular inclusions in the cytoplasm.

Electron microscopy of thin sections revealed that these cytoplasmic elements correspond to the intracytoplasmic viral lesion: the inclusions are the viroplasm and the fine granules, the virions. The intense reaction of the finely granular dense viroplasm with DAPI staining demonstrated the high rate of DNA synthesis. The formation of the virions takes place at the periphery of the viroplasm as previously described (Figure 8).

During the course of development of the viral lesions, the GNV induces progressive hypertrophication of infected cells. Other cytopathological changes are observed as the enlargement of the nucleus combines with a disappearance of the chromatin and the nucleolus. The cytoplasm is also affected by the formation of numerous vacuoles and of dense multilayered spheroidal inclusions, 2–3 μm in diameter (Figure 9).

Mass mortality during the 1970 HIV infection.—Some of the 1970 HIV-affected Portuguese oysters exhibited atrophy and weakness of the adductor muscle; however, no distinctive clinical signs were noted that could be associated with the disease.

The most characteristic histological lesion of the disease is an acute inflammatory response associated with the presence of atypical hemocytes in the connective tissue and an increase in the number of brown cells (Comps 1983). Pycnotic nuclei and basophilic intracytoplasmic inclusion bodies, often with attached groups of fine granules, are found in atypical blood cells. The inclusions (2 or 3 μm in diameter) are round and Feulgen-positive. Acridine orange and DAPI staining demonstrated that the granules and inclusions contain DNA (Figure 10).

Ultrathin sections revealed that these cells were infected with a virus (HIV) (Comps et al. 1976). The inclusions correspond to the virogenic stroma and the granules to the virions. The finely granular viroplasm mass is spheroid in shape. Incomplete virions adhere to the periphery of the viroplasm, whereas detached particles are scattered throughout the cytoplasm. The morphology of the virus was described above and infected cells are shown in Figure 11. The lesion caused by the HIV in the Pacific oyster can be associated with cytoplasmic paracrystalline arrays (Figure 12).

Important Epizootiological and Pathological Aspects

Gill disease (GNV infection).—Gill disease was originally observed by Trochon in the region of La Tremblade in November 1966 (Marteil 1968). The incidence of the disease increased, became epizootic in 1967, and extended to all areas of Portuguese oyster culture in France, including Marennes–Oléron, Bay of Arcachon, Vendée, and some sectors of Brittany. Abnormally high mortalities observed at this time confirmed the virulence of the disease, which reached its highest level in 1967. In most of the affected areas, 70–80% of the Portuguese oysters evidenced gill necrosis. The occurrence of gill disease was also noted in Great Britain, Spain, and Portugal (Ferreira and Dias 1973). After 1968, the disease persisted in an enzootic state in natural stocks of

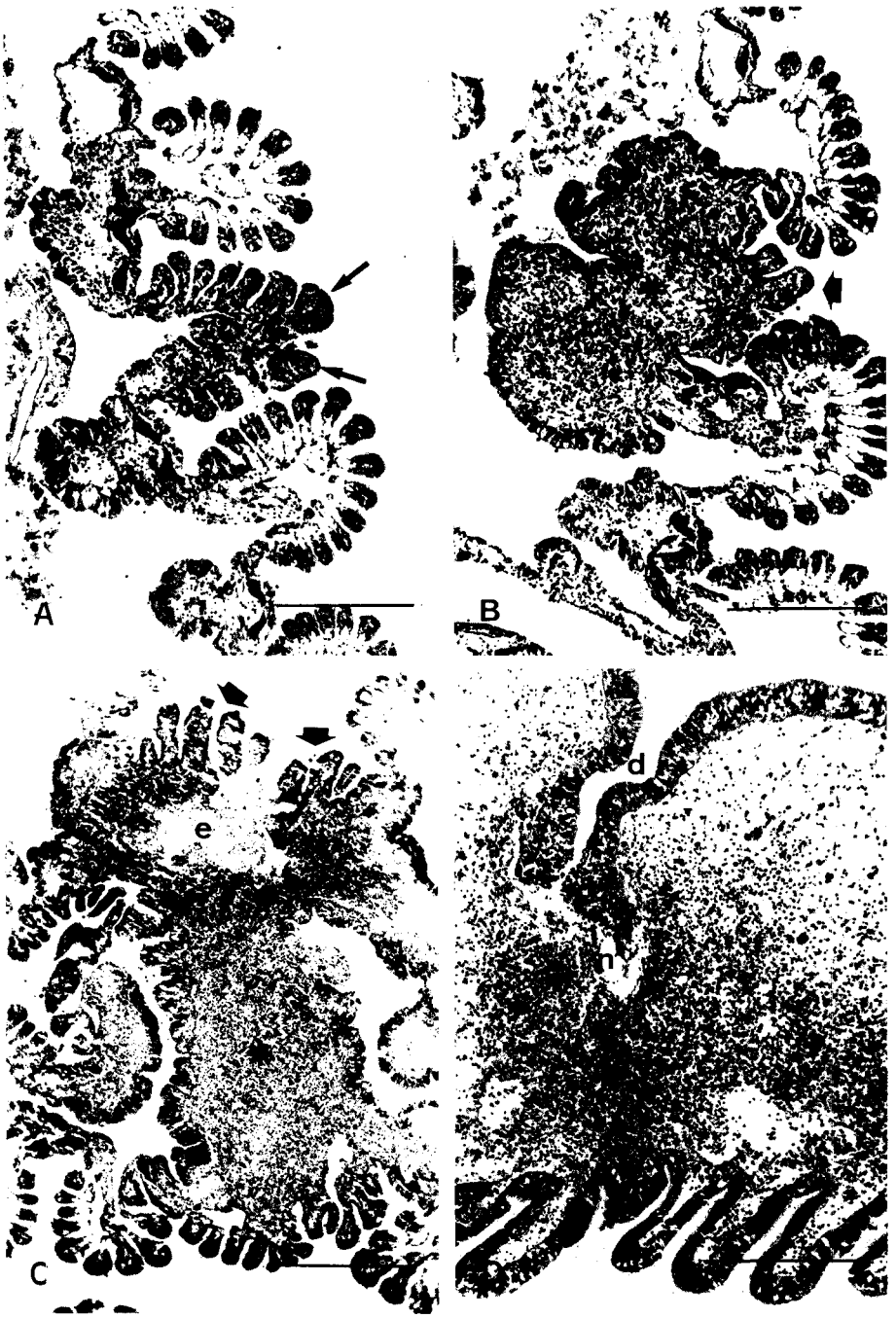

FIGURE 5.—Histopathological changes caused by the gill necrosis virus (GNV) disease. **A.** Early stage of the infection: the inflammatory reaction involves swelling of the gill filaments (arrows). **B.** Advanced stage of the disease: a group of filaments (arrow) is completely destroyed, and the inflammation spreads to the interfilamentar junction (star). **C.** Heavy ulceration of two plicae of filaments (arrows) producing a large excavation (e); in this case, the infection affects also the interlamellar septum (star). **D.** GNV lesion on the labial palp: localized degradation of the epithelium (d) associated with a necrosis of the connective tissue (n); note the intense inflammation around the lesion (star). Bar = 200 μm.

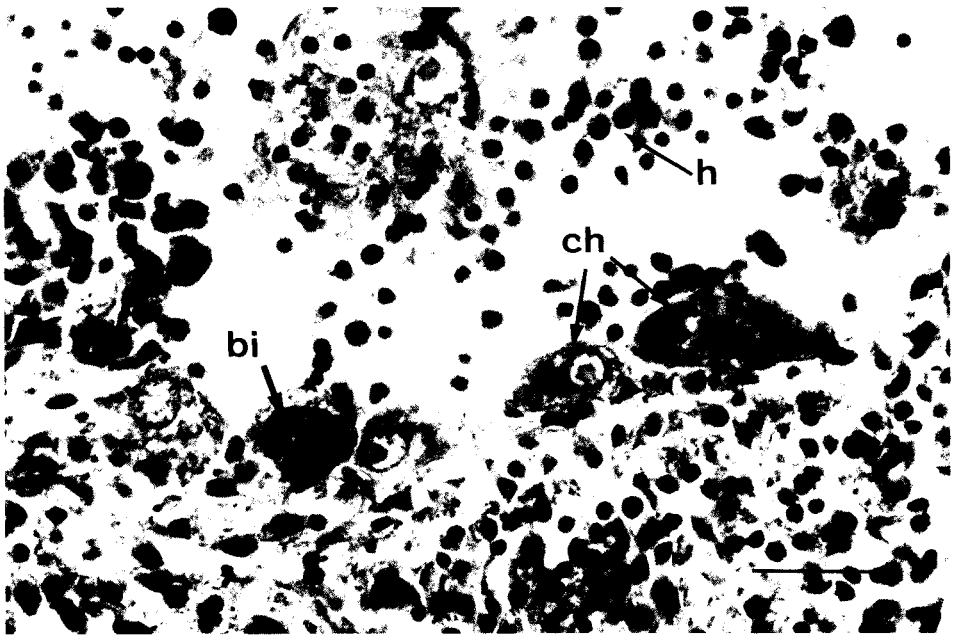

FIGURE 6.—Detail of a gill lesion showing polymorphic hypertrophic cells (ch) and hypertrophic globular cells with basophilic inclusion (bi); hemocytes are scattered around the lesion (h). Azan; bar = 20 μm.

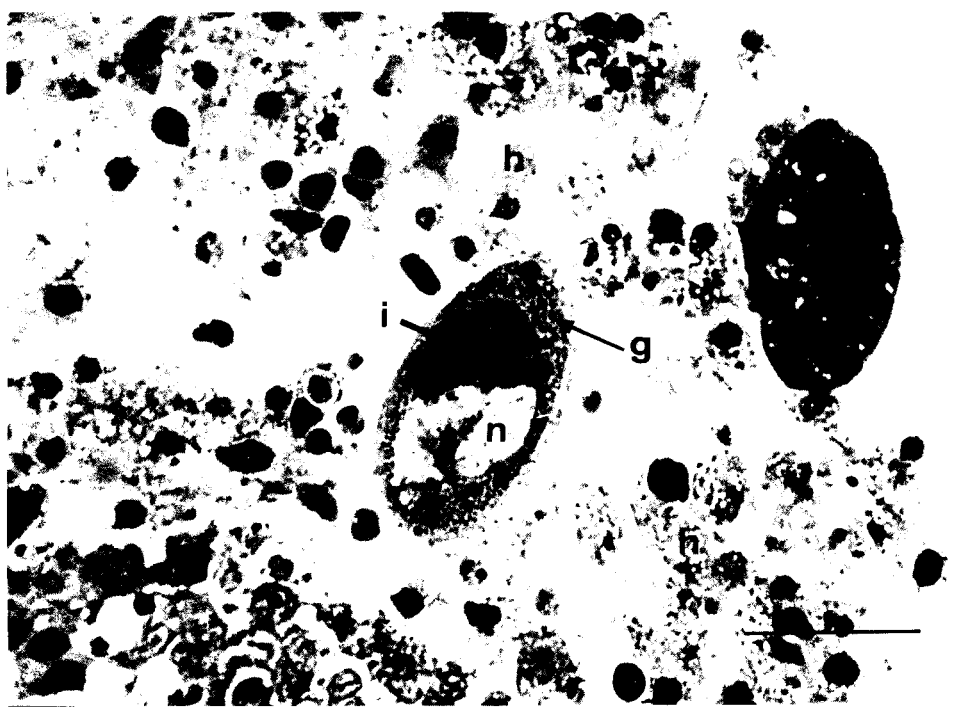

FIGURE 7.—Section of hypertrophic cells infected by gill necrosis virus; n = nucleus; i = basophilic inclusion; g = basophilic granules or virions; h = hemocytes. Toluidine blue; bar = 20 μm.

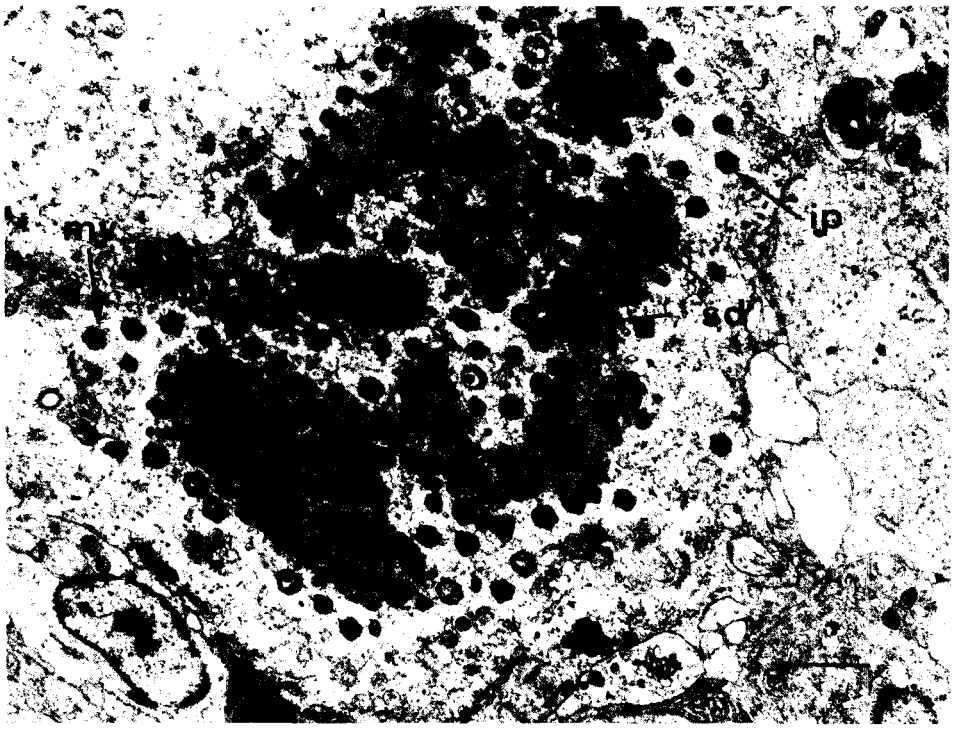

FIGURE 8.—Intracytoplasmic viral lesion in a globular cell; sd = virogenesis according to spots of high density; vs = virogenic stroma; ip = immature particles; mv = mature virions. Electron micrograph; bar = 1 μm.

Portuguese oysters from the Sado River and from the area of Cadiz (Comps and Masso 1978).

Environmental conditions influenced the course of the disease. In the most severe cases, massive necrosis was evident, and destruction of the gills was extensive. Respiratory disorders followed (His 1969), and gametogenesis was interrupted (Marteil 1969). Severe mortality (40%) occurred in Marennes and Bay of Arcachon (Marteil 1968). Surviving Portuguese oysters that were sampled showed healing of gill lesions (Comps 1970).

Historically, some protistans were implicated in necrosis of the gills. Besse (1968) attributed a role to a ciliate of the genus *Trichodina*. Gras (1969) noted the presence of a *Dermocystidium*-like parasite, and Arvy and Franc (1968) ascribed the cause of the disease to a new protistan, *Thanatostrea polymorpha* (Franc and Arvy 1969). However, an ultrastructural study demonstrated that the large cells described as forms of *T. polymorpha* were actually host cells infected with the virus (Comps and Duthoit 1976). The virus was not isolated, and the experimental transmission studies needed to demonstrate its pathogenicity were not undertaken during the progress of the disease. However, numerous histological sections of Portuguese oysters were prepared during the disease epizootics, and a clear relationship was established between the gill lesions and the virus infection.

Mass mortality during the 1970 HIV infection.—Commencing in August 1970, mass mortalities of Portuguese oysters occurred among Marennes–Oléron oysters and in several oyster-producing areas in Brittany. In December, mortalities of this species were reported also in the Bay of Arcachon. The disease spread to other Portuguese oyster culture beds in France with the exception of the Étang de Thau on the Mediterranean coast (Comps 1983). In the south of Spain, recurring mortalities of Portuguese oysters associated with HIV were found in the Gadalquivir River in 1974 (M. Guttierez, Instituto de Investigaciones Pesqueras, Cadiz, personal communication). In 1983, long after the disappearance of the Portuguese oysters in French oyster beds, mortalities due to HIV occurred in Portuguese oyster spat from an experimental hatchery study located in Brittany (Bougrier et al. 1986).

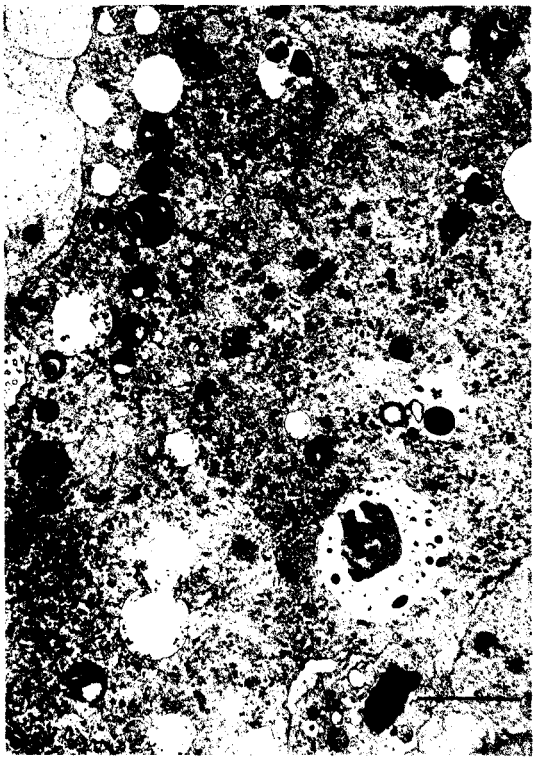

FIGURE 9.—Disorganized cytoplasm of cells infected with gill necrosis virus containing dense multilayered inclusions (i). Electron micrograph; bar = 1 μm.

Oyster Velar Virus Disease (OVVD)

A virus infection of larval Pacific oysters, producing velar lesions and mortalities, has been reported (Leibovitz et al. 1978). Elston and Wilkinson (1985) described the new disease in detail, and they named it oyster velar virus disease. The disease was detected during an 8-year period in the state of Washington in Pacific oysters grown in Wilapa Bay and Puget Sound.

Affected animals were less active and had velar lesions with progressive loss of velar epithelial cells, which then became dissociated, rounded, and detached from the deformed velum. These velar cells contained inclusion bodies and showed viral cellular lesions. DNA was demonstrated by histochemical methods (Feulgen and acridine orange reactions). The intracytoplasmic lesion consisted of a granular, electron-dense viroplasmic inclusion body.

The morphogenesis of the virus particles began with the formation of capsids around the viroplasm, which became irregular in shape. Detached, empty particles had an icosahedral structure. The process of morphogenesis of the viral particles was completed with the formation of the dense viral core. The zone separating the core from the capsids became denser during the process of morphogenesis.

The virions, averaging 228 nm from edge to edge, have capsids 20.6 nm in thickness and are composed of two bilayered membranous structures. The dense inner core, 103 nm × 160 nm, is ovoid in section and is surrounded by a less dense zone.

The importance of this viral infection is evident; however, its relationship to the larval disease has not been established experimentally. Moreover, the seasonal transmission of the disease is difficult to explain, especially as to the mode of transmission.

Discussion

Compared with vertebrates and insects, the knowledge of marine invertebrate virology is very limited and is usually restricted to histopathological and epizootiological data. The viral etiology of the major epizootics of Portuguese oysters was demonstrated in 1976, but research was effectively halted when the susceptible host disappeared from France. Because of this disappearance and the lack of research methodology when the diseases first appeared, the HIV and GNV have not been isolated, purified, or analyzed. Without information on the fundamental characteristics such as density and composition of the polypeptides and nucleic acids, it is not possible to make a serious comparison between the two agents. Presently, they are considered different only on the basis of histological and cytopathological analyses.

The study of OVV, although more recent (Elston and Wilkinson 1985), is also limited to morphological considerations. It has not been isolated, experimentally transmitted, or analyzed biochemically. In spite of these problems, a rough comparison is instructive.

At present, the precise taxonomic positions of HIV and GNV are uncertain, yet they resemble the iridoviruses in general morphology, size, intracytoplasmic morphogenesis, and nucleic acid composition. The lymphocystis virus in fish has recently been classified in the genus *Iridovirus* (Matthews 1979), and the virus causing piscine erythrocytic necrosis (PEN) of marine fish (Atlantic cod *Gadus morhua* and others) is also similar. This group is also a member of the genus *Iridovirus* (Reno et al. 1978; Walker and Sherburne 1977). A further comparison can be made

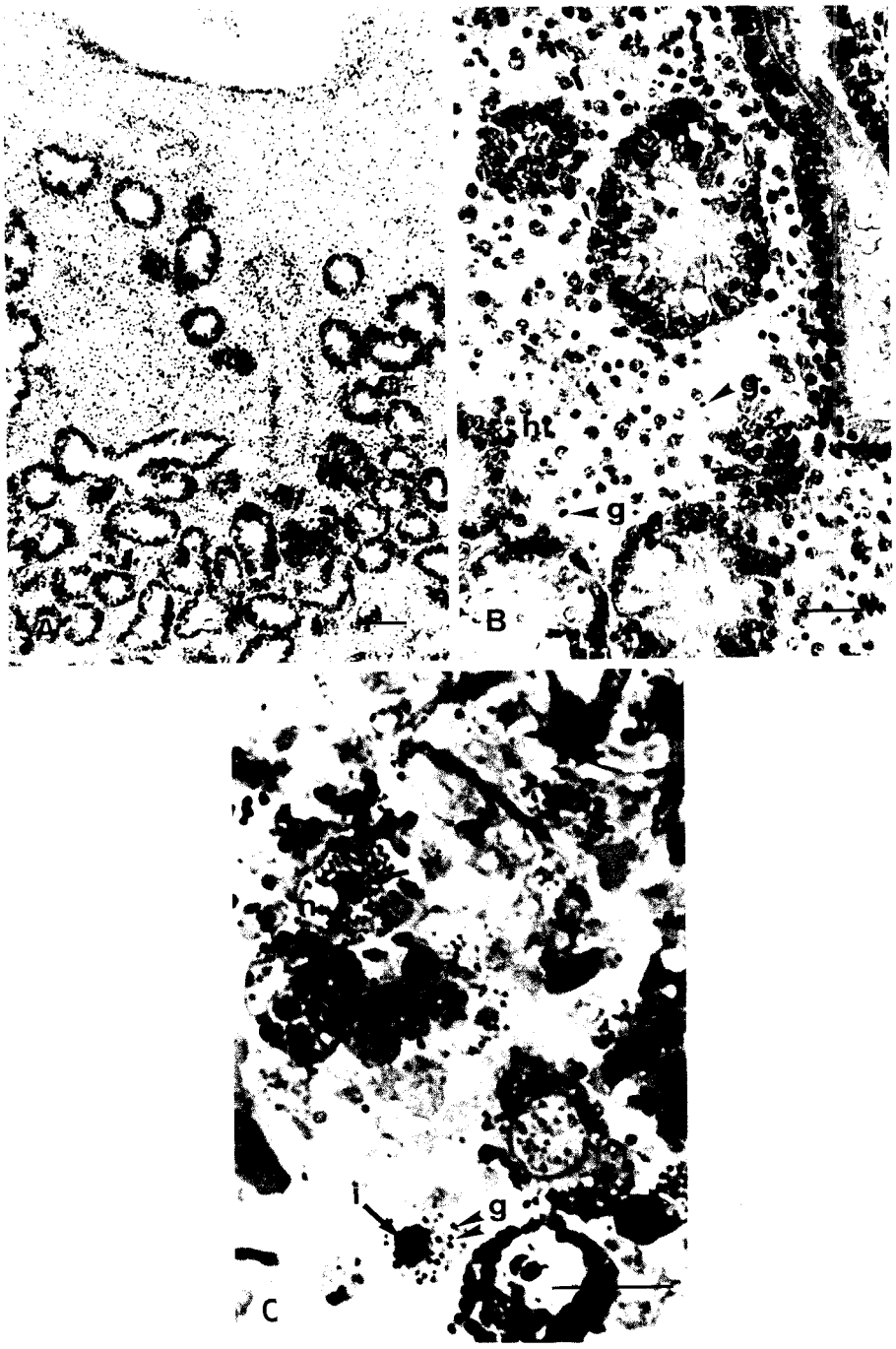

FIGURE 10.—Infection caused by the hemocytic infection virus (HIV). **A.** Characteristic aspect of the connective tissue of an infected Portuguese oyster. Hematoxylin and eosin; bar = 100 μm. **B.** Section of connective tissue showing infected hemocytes (hi) containing basophilic granules (g). Hematoxylin and eosin; bar = 20 μm. **C.** Section through hemocytes with HIV lesion; i = basophilic inclusion or virogenic stroma; g = basophilic granules or virions; n = nucleus showing abnormal aspect. Toluidine blue; bar = 10 μm.

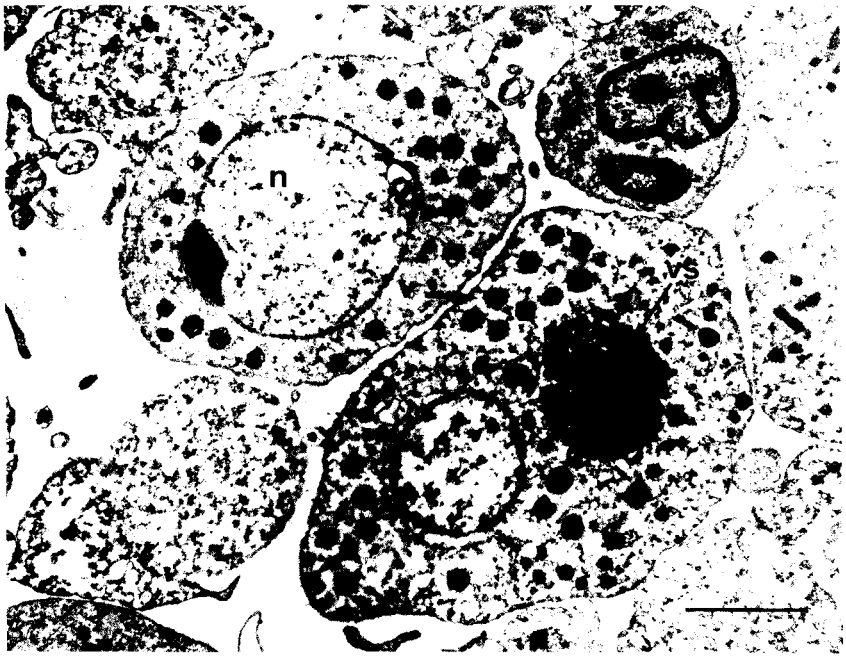

FIGURE 11.—Electron micrograph of cells infected by hemocytic infection virus; n = nucleus; vs = virogenic stroma; v = virions. Bar = 2 μm.

with the virus described by Elston and Wilkinson (1985), which causes OVVD in *C. gigas*. These workers demonstrated similarities in the viral morphogenesis and structures of the virions; however, the viruses differed in some characteristics such as size (228 nm versus 380 nm) and shape of the inner core, which was pararectangular in OVV. Another virus, similar but smaller than OVV (120–140 nm), has been described by Rungger et al. (1971) in the common octopus and classified in the genus *Iridovirus* (Fenner 1976).

Although paracrystalline arrays of virions have been demonstrated in the cytoplasm of some iridovirus-infected cells (Walker and Weissenberg 1965; Devauchelle and Durchon 1973), this characteristic is not a permanent feature of the iridoviruses and has not been observed in the cells of *C. angulata* infected by HIV or GNV. In contrast, the viral lesion caused by HIV, which infects the hemocytes of *C. gigas*, can be associated with paracrystalline inclusions, showing similarities with the inclusion bodies observed during the course of replication of some iridescent viruses (Kelly and Tinsley 1972). Similar inclusions have been reported in the cells infected by an iridovirus causing muscle lesions in *Octopus vulgaris* (Rungger et al. 1971).

If, for the present, the taxonomic position of the GNV and HIV cannot be determined more precisely, both viruses, along with the PEN viruses, the OVV, and some lymphocystis disease viruses, can be considered an original group of marine ICDV.

Another consideration is the host-virus relationship and the susceptibility of both species, *C. angulata* and *C. gigas*, to the pathogen. Other than an exceptional case of HIV and GNV infection found in *C. gigas*, this species, under natural conditions, appears to be highly resistant to these infections. During the summer of 1970 and all of 1971, when Portuguese oysters were greatly affected with mass mortalities, no losses were noted in the Pacific oysters coming from Japan or in the area of Marennes–Oléron. On the contrary, these Pacific oyster-producing areas saw exceptional growth (Comps 1972). Although Pacific and Portuguese oysters are considered the same species by some investigators (Menzel 1974), there is a difference in their susceptibility to HIV.

The ability of the Pacific oyster to resist the 1970 mass mortalities resolved a very serious economic problem. Pacific oyster production was maintained by massive imports of Pacific oyster seed from Japan, Korea, and Canada and by the

FIGURE 12.—Hemocytic infection virus lesion in Pacific oyster showing paracrystalline inclusion (star) associated with virus particles (v). Electron micrograph; bar = 200 nm.

regeneration of the natural Pacific oyster beds with Pacific oysters from British Columbia, Canada. Now, the culture of the Pacific oyster encompasses all the coasts of Europe.

The initial difficulties in isolation and analysis of these viruses stemmed partly from the relative scarcity of the virions. In the GNVD and OVVD, very few cells are infected, and in all cases, the number of virions is light. Similar conditions characterize the erythrocytic infection caused by the large ICDV in Atlantic cod (Reno and Nicholson 1981) and in Atlantic herring *Clupea harengus harengus* (Reno et al. 1978).

The resolution of these problems would be greatly facilitated by the ability to replicate the viruses in vitro in tissue cultures. Some attempts have been conducted with fish cell lines, but the viruses isolated from several species of oysters were not pathogenic. Hill (1976) described three serotypes of viruses isolated from several marine bivalves, particularly from the Pacific and eastern oysters and the edible oyster *Ostrea edulis*, which were cultured in bluegill fry (*Lepomis macrochirus*) cell culture (BF 2). The isolates have identical properties to those of a number of different infectious pancreatic necrosis serotypes. The effects of these viruses on molluscan hosts are unknown.

More recently, a reolike virus designated 13p2, was isolated by Meyers (1979) from juvenile eastern oysters and grown in bluegill fry cell cultures (BG 20). The virus replicated in cell cultures of freshwater bluegill, brown bullhead *Ictalurus nebulosus*, Atlantic salmon *Salmo salar*, guppy *Poecilia reticulata*, and walleye *Stizostedion vitreum*. Experimentally, the virus produced lesions and mortalities in bluegills but was not pathogenic to eastern oysters (Meyers 1980). Clearly, obtaining cell lines from oysters or other marine bivalves is of primary importance to future progress in research on marine molluscan viral diseases.

Acknowledgment

I thank Louis Leibovitz, Laboratory for Marine Animal Health, Marine Biological Laboratory, Woods Hole, Massachusetts, for his suggestions and critical review of the manuscript.

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