A Reo-like Virus Isolated from Juvenile American Oysters (Crassostrea virginica)

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SUMMARY

A filterable agent, designated 13p2 was isolated from homogenized juvenile oyster tissue inoculated on to bluegill fry (BF-2) cell cultures. The oysters were from a hatchery on Long Island Sound, New York. Successive passages resulted in progressive cytopathic effects (c.p.e.) consisting of discrete plaques containing large syncytia seen within 2 to 3 days in cultures held at 15 °C. The agent was concentrated from supernatant fluids by ultracentrifugation. Negative stained preparations examined by electron microscopy revealed icosahedral particles with a mean diam. of 79 nm.

Virus replication in tissue culture occurred at both 15 and 23 °C. Susceptible fish cells in addition to the BF-2 included brown bullhead, Atlantic salmon, guppy embryo and walleye fry lines. Limited c.p.e. occurred in Atlantic salmon heart cells while rainbow trout gonad, rainbow trout spleen and fathead minnow cells were refractory to cytopathic changes.

Biochemical and physical characteristics suggested the 13p2 virus belonged to the family Reoviridae. The possibility that this virus is a known reovirus, present only as a contaminant, was ruled out on the basis of serological results and failure of avian or mammalian cells to support its growth. The 13p2 agent may be an undescribed virus. Further investigations concerning the identity of this virus and its capabilities as a pathogen in fish and shellfish are under way.

INTRODUCTION

There have been several reports of virus or virus-like particles observed in marine mollusc tissues. The first was made by Devauchelle & Vago (1971) who observed virus particles in the stomach cells of the cuttlefish (Sepia officinalis). Other reports followed in which apparent virus particles were seen in muscle cells of Octopus vulgaris (Rungger et al. 1971), in the nuclei of cells in the American oyster, Crassostrea virginica (Farley et al. 1972) and in the cytoplasm of atypical cells in the Portuguese oyster, C. angulata (Comps et al. 1976). However, these reports were based only upon electron microscopic examination of field-collected material from which virus isolations were either not attempted or unsuccessful. In 1973, Buchanan reported paracrystalline arrays of virus particles in the cytoplasm of secretory cells in the digestive gland of Tellina tenuis. Later, Hill (1976a) reported the first tissue culture isolation of a molluscan virus from digestive glands of Tellina clams. The virus was isolated in the bluegill fry (BF-2) cell line (Wolf et al. 1966) and morphologically resembled the infectious pancreatic necrosis (IPN) virus (Wolf et al. 1960) of salmonid
fish. Subsequent work revealed that Hill's virus was not the same as that observed in *Tellina* by Buchanan (1978). Other isolates of the virus were obtained from wild populations of various bivalve molluscs including the Japanese oyster (*C. gigas*), European oyster (*Ostrea edulis*), hard clam (*Mercenaria mercenaria*) and from the following gastropods: limpets (*Patella vulgata*) and winkles (*Littorina littorea*) (Hill, 1976b).

This report describes the isolation in tissue culture of a virus from juvenile North American oysters which appears to be different from the virus isolated by Hill (1976a, b).

**METHODS**

*Cell cultures and media.* The BF-2 cell line was used for the primary isolation of the 13p2 virus. Fish cells used to test the host range of the virus included the following cell lines (obtained from the American Type Culture Collection, Rockville, Md., or through the courtesy of Dr Ken Wolf): brown bullhead (BB; Cerini & Malsberger, 1962), rainbow trout gonad (RTG-2; Wolf & Quimby, 1962) and fathead minnow (FHM; Gravell & Malsberger, 1965). Other fish cells tested included the Atlantic salmon (AS, Nicholson & Byrne, 1973), guppy embryo (GE-4; B. W. Calnek, unpublished data), walleye fry (WF-2; B. W. Calnek, unpublished data), Atlantic salmon heart (ASH; V. Lund, unpublished data) and rainbow trout spleen (RTS, R. Elston, unpublished data) lines. Primary chicken kidney cell (CKC) cultures prepared from specific-pathogen-free (SPF) 2- to 3-week-old chickens as described by Calnek & Madin (1969) were also tested for susceptibility to this agent. Six mammalian cell lines were tested for their susceptibility to the 13p2 virus. These cell lines (kindly provided by Dr Max Appel, Baker Institute, Cornell University, Ithaca, N.Y.) included canine kidney (MDCK), rabbit kidney (RK), porcine kidney (PK), green monkey kidney (VERO), foetal bovine spleen (FBS) and lamb kidney (LK). The BF-2, BB, GE-4 and FHM cells were grown in 'FM-1' medium consisting of Earle's base and Eagle's minimal essential medium (MEM) with added bicarbonate and tris buffers, 10% bovine foetal serum (BFS) and 100 international units/ml of penicillin, streptomycin and 25 μg/ml of amphotericin B. All other fish cell lines were grown in 'FM-4' medium which differed from FM-1 in that Hanks' base MEM was used and there was no tris buffer. All mammalian cells were grown in medium 199 as described for CKC cultures (Calnek & Madin, 1969). Fish and mammalian cells were grown at 23 and 37 °C, respectively, in small plastic flasks (25 cm²).

*Source of materials and virus isolation.* A survey of shellfish diseases of Long Island oysters and hard clams (*Mercenaria mercenaria*) included screening both molluscan species for indigenous viruses. A total of 72 juvenile (6 to 8 months) and 139 adult hard clams from three Long Island hatcheries were examined between November 1976 and September 1977. During the same period, 202 juvenile and 58 adult oysters from three hatcheries were also screened. A total of five different hatcheries on both the north and south shores of Long Island were represented in this study. Juvenile clams and oysters were taken directly from hatchery facilities while hatchery-reared adults were harvested from leased bottoms.

Whole animals were excised, pooled and homogenized in 3 vol. of modified FM-1 medium (2% rather than 10% BFS). Each sample usually was divided into two pools and the number of animals per pool ranged from 5 to 10 for adult oysters and clams and 12 to 20 for juveniles, depending on their sizes. The homogenized suspension was centrifuged at 1500 g for 20 min. The supernatant was diluted 1:10 in FM-1 and passed through a serum-soaked 0.45 μm membrane filter. Five ml of resulting extract was inoculated directly onto drained 24 to 48 h monolayers of BF-2 cells and incubated at 15 °C for 7 days. The cultures were then harvested by shaking the cells loose from the flask into the medium and the cells disrupted by sonic
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vibration for 20 s (Model W-140D Sonifier Cell Disrupter, Heat Systems-Ultrasonics, Plainview, N.Y.). The suspension was diluted 1:10 in FM-1 and passaged by inoculating 5 ml on to fresh, drained BF-2 monolayer cultures. Two blind passages were made before samples were considered negative. Cell cultures showing c.p.e. were held until there were extensive changes before harvesting. Passage of 'positive' samples was as above except that the suspension was also passed through a 0.2 µm membrane filter to eliminate any possibility that the cellular changes were caused by a bacterial contaminant.

Virus titrations were performed as described by Hoskins (1967); tissue culture 50% infective dose endpoints (TCID₅₀) were determined according to the method of Reed & Muench (1938). A working stock of tissue culture propagated virus with an approximate titre of 3 × 10⁷ TCID₅₀/ml was stored at -70 °C and used for all titrations and host-range studies in fish cells and CKC cultures. All fish cell cultures used in the susceptibility studies were inoculated with 500 TCID₅₀ of virus while CKC cultures received 5 ml of 10⁶ TCID₅₀/ml. Mammalian cell cultures each received a virus dosage of 0.1 ml of 10⁶ TCID₅₀/ml. In all host range experiments, susceptible BF-2 cell cultures were inoculated in parallel as positive 13p₂ virus controls.

Virus characterization. For examination of negative-stained virions, the virus was concentrated from supernatant fluids of infected BF-2 cells by centrifugation at 81 500 g for 3 h. Virus pellets were resuspended in double-distilled water and negatively stained for 1 min with 2% phosphotungstic acid at pH 7.0 on Formvar coated copper grids. These preparations were examined in an Hitachi HU-11E electron microscope. Virus particles were measured using an internal control of bovine liver catalase crystals according to the method of Wrigley (1968).

Infected cells for examination by thin section electron microscopy (EM) were scraped free from the culture vessel with a rubber policeman. Harvested cells were fixed for 2 h in 4% glutaraldehyde with 0.1 M-sodium cacodylate buffer at pH 7.6 and post-fixed for 1 h at 4 °C in cacodylate buffered 2% osmium tetroxide. Samples were embedded in Epon-Araldite plastic for thin sectioning.

Serological characteristics. Neutralization tests with 13p₂ virus were performed using a rabbit polyvalent IPN antiserum no. 149. The method used was the constant virus, varying antiserum dilution procedure described by Schmidt & Lennette (1965). Doubling antiserum dilutions from 1/10 to 1/2560 were each reacted against 100 TCID₅₀ of virus and inoculated into six BF-2 cultures. The virus was first passed through a 0.2 µm filter to remove any large aggregates of particles before titration of stock material. Control cultures were inoculated with test dilutions of 13p₂ virus and IPN virus in 1/10 normal rabbit serum and IPN virus reacted with IPN antiserum dilutions of 1/10 and 1/160. Similar neutralization tests were conducted using a rabbit Tellina virus antiserum with a titre of 1/2000000. Serum neutralization tests were also performed using antisera against feline reovirus type 3, canine reovirus type 1 and chicken FDO reovirus. As positive antiserum controls, parallel neutralization tests were done using the same feline, canine and avian antisera against their homologous reoviruses. These tests were carried out in cell cultures of feline embryo fibroblasts (FEF), VERO and CKC, respectively. Serum antibody titres were determined on the basis of the highest dilution which neutralized virus as evidenced by lack of c.p.e. in the inoculated cultures.

The ability of the 13p₂ virus to haemagglutinate human type O erythrocytes at 4 and 37 °C was tested according to the methods of Hoskins (1967). The protocol was altered by incorporating microtitre procedures in which 0.1 ml quantities of saline, erythrocyte suspension and virus were used. The 13p₂ test material consisted of tissue culture fluid
having a titre of \(3.16 \times 10^2\) TCID\(_{50}\)/ml and virus concentrated 100-fold by ultracentrifugation. Tissue culture fluids containing a paramyxovirus (5.0 \(\times\) \(10^6\) TCID\(_{50}\)/ml) recently isolated and characterized from psittacine birds (K. Hirai, unpublished data) and Newcastle disease virus (9.95 \(\times\) \(10^5\) TCID\(_{50}\)/ml), were used as positive controls at 37 and 4 °C, respectively. Additional positive controls consisted of canine reovirus type 1 (1.79 \(\times\) \(10^7\) TCID\(_{50}\)/ml) and feline reovirus type 3 (1 \(\times\) \(10^8\) TCID\(_{50}\)/ml) tested at both temperatures.

Chemical and physical characterization. Chloroform sensitivity tests were performed using the procedure of Feldman & Wang (1961). Briefly, virus suspensions treated with chloroform were titrated along with untreated control suspensions. Loss of infectivity greater than 1 log\(_{10}\) was evidence of susceptibility to the treatment. Channel catfish virus (CCV), which is an enveloped herpesvirus (Fijan, 1968) served as a control. The CCV is specific for ictalurid fish, therefore, all titrations with that virus were done using the BB cell line.

Alkaline and acid stability of the \(13p_2\) virus was determined by titration in BF-2 cells following exposure of the virus to pH 2, 3 or 9 for 30 min at 4 °C. The virus was considered stable if the titre remained unchanged or dropped less than 1 log\(_{10}\) when compared to control titrations of untreated virus (Hamparian et al. 1963).

Cell cultures treated or untreated with the DNA inhibitor 5-iodo-2-deoxyuridine (IdUrd), were infected with virus to determine the type of nucleic acid in the \(13p_2\) isolate. CCV was again used as a control virus. For treatment, maintenance medium containing 35 \(\mu\)g/ml of IdUrd was placed on monolayers of BF-2 and BB cells overnight. The cultures were then drained and inoculated with virus dilutions. New medium (containing IdUrd for treated cultures) was added. A drop in titre of 1 log\(_{10}\) or more would indicate inhibition of virus replication and therefore a DNA virus.

Preliminary studies were conducted to determine the optimum temperature for production of virus c.p.e. BF-2 cultures inoculated with 500 TCID\(_{50}\) of virus were incubated at 15, 23 and 30 °C along with uninoculated controls.

RESULTS

Virus isolation

A progressive c.p.e. developed within 6 days in a BF-2 cell culture inoculated with first-passage material prepared from a single pool of seven juvenile American oysters (\(C.\) virginica) collected in March 1977. A second pool of seven juveniles from the same sample was negative. The c.p.e. consisted of discrete plaques containing large central syncytia (Fig. 1 a, b). These plaques progressed until the entire monolayer became involved. Passage of this material on to a fresh BF-2 cell culture resulted in a similar c.p.e. within 3 days of incubation. This isolate was designated \(13p_2\). A second isolate producing apparently identical type c.p.e. was obtained from both pools, each with 12 juvenile oysters, from a sample collected from a different hatchery in August, 1977. Based on the characteristic c.p.e., this second isolate was thought likely to be the same as \(13p_2\); however, no further characterization was done. Both hatcheries yielding the positive samples were located on embayments along the north shore of Long Island Sound and were the only sources from which juvenile oysters were screened for viruses.

Virus characterization

Abundant icosahedral particles with a mean diam. of 79 nm were observed in the negative stained preparations (Fig. 1 c). The mean, having a standard error of 0.666, was computed from 44 particles measured at the longest axis. Some particles had definite hexagonal pro-
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Fig. 1. Cytopathic effect of \(13\)P\(_4\) virus in (a) living (courtesy of Dr K. Schat) and (b) Giemsa stained BF-2 cells; (c) electron micrographs of \(13\)P\(_4\) particles in thick stain; (d, f) mature particles in the cytoplasm of an infected BF-2 cell; (e) a group of partially formed particles in the cytoplasm of an infected BF-2 cell.
files and an inner capsid layer. However, most appeared slightly oval with clear spike-like projections on the outer capsid and a distinct inner core.

A chronological examination of virus infected BF-2 cells by EM was done by infecting cells with 0-25 ml of undiluted virus stock and harvesting at 4 h intervals from 1 to 44 h post inoculation (p.i.) and then at 51 h p.i. In this study, plaque formation first occurred at 36 h p.i. However, thin sections from the harvested cell material revealed very few virus particles until 51 h p.i. Virions were scattered throughout the cytoplasm of infected BF-2 cells. Each particle was rounded with two capsid layers and an electron dense core (Fig. 1 d, f). Faint particle outlines, which may have represented partially formed virus, were observed in small arrays within the cytoplasm (Fig. 1 e).

The 13p2 virus was not neutralized by any of the dilutions of either polyvalent IPN or Tellina virus antisera tested. The same IPN antiserum had a titre of $\geq 1:160$ against IPN virus. No neutralization of the 13p2 virus occurred with any of the dilutions of the reovirus antisera used. Positive control neutralization tests with homologous reoviruses indicated titres of 1/320, 1/640 and 1/160 for the same canine, feline and avian antisera, respectively.

Haemagglutination results were negative for both preparations of the 13p2 virus tested. Tissue culture fluids of the psittacine paramyxovirus and of the Newcastle disease virus used as positive controls had one haemagglutinating (HA) unit at dilutions of 1/64 and 1/8, respectively. Results for reovirus controls were as follows: type 3 had one HA unit at dilutions of 1/16 and 1/8 at 37 and 4 °C, respectively. Type 1 had one HA unit at a dilution of 1/32 for both temperatures. Saline and cell culture media controls were negative.

The results of the physical and chemical tests conducted with the 13p2 virus are summarized in Table 1. Virus infectivity was unaffected by chloroform treatment as expected since the electron micrographs did not suggest the presence of a virus particle envelope. Similarly treated CCV was completely inactivated confirming the efficacy of the treatment. The virus was stable at pH 3 and pH 9 but not at pH 2. For unexpected reasons, the virus titre increased by 3 log_{10} after treatment at pH 3. This result was not thought to be spurious because a replicate trial done at a later date gave essentially identical results. This finding may have been due to the breaking up of large aggregates of particles at low pH.

In the presence of the metabolic inhibitor, IdUrd, no significant loss of virus titre occurred in either BF-2 or BB cell cultures. Growth of CCV, as evidenced by c.p.e., was completely

<table>
<thead>
<tr>
<th>Virus tested</th>
<th>Treatment</th>
<th>Assay culture*</th>
<th>Treated virus</th>
<th>Untreated virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>13p2</td>
<td>IdUrd</td>
<td>BF-2</td>
<td>1:42 × 10^4</td>
<td>3:16 × 10^7</td>
</tr>
<tr>
<td></td>
<td>IdUrd</td>
<td>BB</td>
<td>5:57 × 10^4</td>
<td>3:16 × 10^6</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>BB</td>
<td>1:40 × 10^6</td>
<td>4:70 × 10^6</td>
</tr>
<tr>
<td></td>
<td>pH 3</td>
<td>BF-2</td>
<td>2:40 × 10^4</td>
<td>3:90 × 10^6</td>
</tr>
<tr>
<td></td>
<td>pH 9</td>
<td>BF-2</td>
<td>4:90 × 10^2</td>
<td>3:90 × 10^6</td>
</tr>
<tr>
<td></td>
<td>-70 °C, 30 days</td>
<td>BF-2</td>
<td>3:16 × 10^7</td>
<td>4:41 × 10^7</td>
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<tr>
<td></td>
<td>4 °C, 30 days</td>
<td>BF-2</td>
<td>6:40 × 10^7</td>
<td>3:16 × 10^7</td>
</tr>
<tr>
<td>CCV</td>
<td>IdUrd</td>
<td>BB</td>
<td>0</td>
<td>5:57 × 10^3</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>BB</td>
<td>0</td>
<td>7:40 × 10^2</td>
</tr>
</tbody>
</table>

* BB = brown bullhead; BF-2 = bluegill fry.† Replicate trials.

Table 1. The stability of the 13p2 virus and the channel catfish virus (CCV) after various physical and chemical treatments
Table 2. Cytopathic schedule of fish cell lines inoculated with 500 TCID₅₀ of 13p₂ virus incubated at 15 °C

<table>
<thead>
<tr>
<th>Fish cell line</th>
<th>Days post-infection c.p.e. first observed</th>
<th>Days post-infection c.p.e. completed (4+)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bluegill fry</td>
<td>2</td>
<td>8 to 10</td>
</tr>
<tr>
<td>Brown bullhead</td>
<td>3-5</td>
<td>8</td>
</tr>
<tr>
<td>Guppy embryo</td>
<td>7-12</td>
<td>28 to 30</td>
</tr>
<tr>
<td>Atlantic salmon</td>
<td>3-5</td>
<td>12 to 13</td>
</tr>
<tr>
<td>Walleye fry</td>
<td>3-4</td>
<td>10 to 13</td>
</tr>
<tr>
<td>Atlantic salmon heart</td>
<td>5</td>
<td>Few plaques - do not progress up to 30 days</td>
</tr>
<tr>
<td>Fathead minnow</td>
<td>—†</td>
<td>—</td>
</tr>
<tr>
<td>Rainbow trout gonad</td>
<td>—†</td>
<td>—</td>
</tr>
<tr>
<td>Rainbow trout spleen</td>
<td>—†</td>
<td>—</td>
</tr>
</tbody>
</table>

* Total c.p.e. involvement of the cell monolayer.
† No c.p.e. detected during a 30-day observation period.

inhibited by the same IdUrd treatment. The titre of the 13p₂ virus also remained stable following storage at −70 or 4 °C for at least 30 days.

Rate of development of c.p.e. in BF-2 cell cultures varied at different temperatures depending upon the passage of virus used. At 15 °C, low passage virus (3rd passage) produced complete c.p.e. (4+) within 3 days p.i. At 23 °C, 4+ c.p.e. did not occur until 10 to 14 days after inoculation of the same dosage. With higher passage virus (12th passage), the time required for development of c.p.e. was the same for cultures incubated at 15 and 23 °C. No c.p.e. occurred in cultures inoculated at 30 °C regardless of the virus passage. Since BF-2 cells do not survive well at 9 °C, AS cells, which are susceptible but low-temperature tolerant, were used to test the development of c.p.e. at this temperature. After inoculation with 500 TCID₅₀ of virus and incubation at 9 °C for 60 days, no c.p.e. was observed. The inoculated cell culture was moved to 15 °C and typical plaques appeared after 5 days. Similarly treated uninfected cultures were normal in appearance.

Host range in vitro

Results of the fish cell host-range studies are in Table 2. Cultures other than BF-2 which were found to be susceptible to the 13p₂ virus included the BB, AS, GE-4, WF-2 and ASH lines. RTG-2, RTS and FHM cells were refractory; i.e. there were no cytopathic changes after 30 days of observation. In susceptible cultures, plaques appeared at different times, depending on the cell line used and progressed until 4+ involvement of the monolayer. An exception was the ASH line in which only a few plaques appeared; these remained but failed to progress appreciably in growth. Inoculated ASH and RTS cells were kept at 15 °C for 75 and 100 days respectively, after which many cells were rounded and transparent with large numbers detached in the supernatant fluids. Similar apparently degenerative changes were seen in uninoculated control cultures held at 15 °C, but generally not as early as in inoculated cultures. No plaques were evident. Both control and infected cultures were harvested, diluted 1:10 and assayed on fresh BF-2 cultures. Typical plaques developed by 3 days p.i. in the cell cultures receiving infected ASH and RTS cells.

No c.p.e. was observed in inoculated CKC cultures during 9 days of incubation at 38.5 °C. Control BF-2 cultures inoculated in parallel developed severe c.p.e. 4 to 6 days p.i. at 15 °C. CKC cells were trypsinized and replated for a second passage. Thereafter, six
additional passages were made, each with 5 ml of a 1:10 dilution of whole kidney cells and supernatant inoculated onto fresh CKC cultures. At each passage, cultures of the inoculated line became more acid than did those of the uninoculated line. During the 5th and 7th passages, questionable changes were observed which consisted of generalized thinning of cells and development of ‘holes’ in the monolayers. Whole kidney cells and supernatants from the inoculated line were harvested from the 1st, 3rd, 5th and 7th passages; 1/10 of the yield from each culture was inoculated onto a 24 to 48 h BF-2 cell culture. No c.p.e. appeared at the end of 7 days. A second passage was then made on to fresh BF-2 cultures; virus was recovered only from flasks inoculated with the first passage CKC culture material. All other cultures were negative.

By 4 days p.i., positive control BF-2 cells receiving the same virus dose as mammalian cells were completely destroyed by the 13p₂ agent. Inoculated mammalian cells appeared more acid than control cultures. By 6 days p.i. severe cellular degeneration was observed in the inoculated FBS culture. Cells were rounded and granular in appearance with many floating free in the supernatant. Monolayers of inoculated LK cells appeared thinned. Supernatants from all inoculated and control mammalian cell cultures were passed on to fresh monolayers by allowing 0-8 ml to adsorb for 1 h at room temperature. Supernatants from both control and inoculated FBS and LK cells were also passed on to 48 h monolayers of BF-2 fish cells. Continued observation of first passage cell cultures revealed no additional changes after 14 days. Increased acidity of inoculated mammalian cell cultures was observed again during the second passage. After 14 days, no c.p.e. was observed in any cultures except FBS cells. At 6 days p.i., FBS cells developed degenerative changes similar to those seen in first passage. The supernatant was harvested, filtered through a 0-2 μm membrane and passed on to a fresh FBS cell culture. After 14 days of observation, no c.p.e. appeared and the usual increased acidity of inoculated cultures was not apparent. Th: BF-2 cultures receiving first passage supernatants from degenerated FBS and LK cells showed no c.p.e. after 9 days. Cells were shaken from the flasks, sonicated and passed as before on to fresh BF-2 cultures. By 5 days p.i. these fish cell cultures developed plaques typical of the 13p₂ virus. The supernatant from inoculated second passage VERO cells was also passaged twice on BF-2 cell cultures but no virus was recovered.

DISCUSSION

Among all the samples of hard clams and American oysters screened for viruses, only two samples of juvenile oysters yielded cytopathogenic agents. While the c.p.e. type suggested that they may be identical, definition of this point must await further testing.

These positive samples from two pools of 7 and 24 animals, respectively, were collected five months apart from two different hatcheries. Not enough samples were taken from either hatchery to determine individual site prevalences, nor was enough information available to determine if the prevalence of this virus was seasonally influenced.

Among the fish cell lines tested, those which were susceptible represent taxonomic families which are widely divergent. This infers that the 13p₂ virus is not highly specific in its host cell requirements when cultured in vitro. For the purposes of in vitro culture, the BF-2 and BB cell lines appeared most suitable in terms of virus susceptibility. The diffuse degeneration observed in inoculated ASH and RTS cells kept over long periods of time were probably normal changes of senility which may have occurred earlier in cells metabolically altered by adsorbed virus or otherwise affected by the inoculum materials. That infectious virus was retrievable was shown by re-isolation in BF-2 cell cultures inoculated with infected ASH and
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RTS cells. Virus recovery from ASH cells was not surprising since virus replication obviously occurred at low levels as shown by slight c.p.e. still observable at 30 days. RTS cells were refractory to c.p.e. but whether virus actually replicated or just survived in the medium cannot be answered at present.

The suspicious cellular changes observed in the 5th and 7th passages of virus inoculated CKC were considered to be unrelated to the 13p2 virus since no virus was recovered after the first passage; that single recovery was probably due to residual inoculum virus.

Among the mammalian cell lines tested, only the FBS line appeared to be susceptible to the 13p2 virus. However, the c.p.e. observed in the first and second passages of virus inoculated FBS cells was considered to be unrelated to the 13p2 virus for three reasons: (a) the c.p.e. observed was not typical of that described for the 13p2 virus; (b) increased acidity did not occur nor was c.p.e. reproducible after filtration of passaged material; (c) the 13p2 virus could not be recovered following a second passage on mammalian cells. Recovery from first passage material was again probably due to residual inoculum and it is likely that a contaminant in the virus inoculum was responsible for the cellular and pH changes observed.

The negative neutralization tests with IPN antiserum and 13p2 virus together with the features of 13p2 virus in terms of its size, capsid morphology, c.p.e. and host susceptibility indicate that this virus is not IPN. For similar reasons it is unlikely to be a strain of Tellina virus.

Several of the characteristics determined thus far for 13p2 are shared by viruses belonging to the family Reoviridae; e.g. it probably contains RNA, it lacks an envelope, it is acid stable at pH 3 and falls within the proper size range. However, the following characteristics suggest that the 13p2 virus is different from other known reoviruses. It does not haemagglutinate human type O erythrocytes and is not neutralized by avian reovirus antiserum or by antisera against animal reovirus types 1 and 3. It does not produce c.p.e. in mammalian or avian kidney cells which support the growth of other known animal reoviruses. Therefore, the possibility is very remote that the 13p2 virus is a known reovirus contaminant concentrated by oysters from the surrounding sea water. A more plausible explanation is that this virus is new and undescribed. The susceptibility of fish cells and the psychrophilic nature of the virus suggest an origin in either aquatic invertebrates or fish. Whether the 13p2 virus can replicate and produce pathological conditions within living fish or shellfish is under investigation.

I wish to thank Mr R. Elston for providing the RTS cells and Dr B. W. Calnek for the WF-2 and GE-4 cells. I am grateful to the following people for their contributions of antisera and homologous viruses: Dr Max Appel, canine type I reagents; Dr Frederick Scott, feline type 3 reagents and FEF cells; Dr B. W. Calnek, avian FDO reagents; Dr Ken Wolf, IPN reagents. I offer special thanks to Dr B. J. Hill for his candid, helpful exchanges of information and ideas through the course of this work and for his contribution of Tellina virus antiserum.

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