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ULTRASTRUCTURAL, BIOCHEMICAL AND BIOPHYSICAL PROPERTIES OF AN ERYTHROCYTIC VIRUS OF FROGS FROM ONTARIO, CANADA

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ABSTRACT: Frog erythrocytic virus (FEV), one of the largest icosahedral viruses, is enveloped, measures up to 450 nm in diameter, and contains double stranded DNA. The virus is found in the cytoplasm of erythrocytes of *Rana catesbeiana*, *Rana septentrionalis*, and *Rana clamitans* from Algonquin Park, Ontario (Canada). Acidophilic inclusions in infected erythrocytes stained with Giemsa's stain correspond to viroplasms from which FEV buds and forms aggregates of virus particles as seen in the electron microscope. Frog erythrocytic virus appears to acquire its envelope from lamellar membranes which surround the virus particles. The virus is structurally sensitive to cesium chloride, potassium tartrate and glycerol. It is also sensitive at pH 1 to 5 and a temperature of 56 C for 15 min. The virus contains at least 16 proteins which range in relative molecular mass from 19.5 to 91.0 kilodaltons (kDa), with two major proteins of 31.0 and 43.0 kDa. The viral DNA has a buoyant density of 1.690 ± 0.005 g/ml, guanine plus cytosine ratio of 25 to 36%, and a melting temperature of 82 to 86 C. Data from this study indicate that FEV should be included in the family Iridoviridae.

Key words: Frog erythrocytic virus, *Rana* spp., ultrastructure, biochemical and biophysical properties, characterization, iridovirus.

INTRODUCTION

Intraerythrocytic inclusions were first recorded from amphibians and reptiles by Labbé (1894) and subsequently have been described as different protozoans or even artifacts (Laveran, 1903; Billet, 1904; França, 1911). Electron microscopy revealed that these inclusions contained viruses which measured up to 380 nm in diameter. Some of the largest were reported in frogs (Bernard et al., 1968; Johnston, 1975; Sousa and Weigl, 1976; Desser and Barta, 1984). All intraerythrocytic viruses are icosahedral, contain double stranded DNA and are confined to the cell cytoplasm; some are enveloped. Because of their large size and icosahedral symmetry these viruses have been included presumptively in the family Iridoviridae (Reno et al., 1978).

Aside from their ultrastructural characteristics, little is known about the intraerythrocytic viruses. In this study, we report on the ultrastructural and certain biochemical and biophysical properties of an intraerythrocytic virus in bullfrogs (*Rana catesbeiana*), mink frogs (*Rana septentrionalis*) and green frogs (*Rana clamitans*) from Algonquin Park, Ontario, and classify the virus based on these features.

METHODS

Hosts

Juvenile and adult bullfrogs (n = 1,762), mink frogs (n = 1,040) and green frogs (n = 709) were caught at the Wildlife Research Station in Algonquin Provincial Park, Ontario (45°35′N, 78°30′W) during mid-May to mid-September of 1985 to 1987. *Rana pipiens* (leopard frogs) (n = 30) were acquired from Boreal Inc. (Mississauga, Ontario, Canada L4X 1K3). The frogs were transported to the University of Toronto (Toronto, Ontario, Canada M5S 1A1), where they were kept in plastic cages containing tap water and fed earthworms. The room in which the frogs were kept was maintained at 10 C and a 12 hr light cycle. Blood was collected from the maxillary vein of frogs with a syringe containing heparin sodium (Hepalean®, Organon Inc., Toronto, Canada M5S 1A1).

Light microscopy and histochemistry

Thin blood smears were air dried, fixed with methanol, stained with Giemsa's stain in phosphate buffer, pH 7.2, and examined for erythrocytic cytoplasmic inclusions. Erythrocytes containing inclusions and those lacking inclusions were measured using an ocular microm-

eter. All measurements are given in μm as a range followed by a mean, standard deviation and sample size. The nucleic acid of the virus was characterized by two methods. Smears of both uninfected and infected frog blood were stained with acridine orange as described by Hansen et al. (1970) and examined by fluorescent microscopy or stained with Feulgen stain as described by Kuchler (1977).

Ultrastructure

Infected blood was fixed in Sorensen's phosphate buffered 2.5% (v/v) glutaraldehyde, postfixed in 1% osmium tetroxide (in the same buffer) and processed for transmission electron microscopy according to the method of Barta et al. (1987). All observations were made with a Philips 201C electron microscope (Philips Inc., Eindhoven, The Netherlands 5600) at an accelerating voltage of 60 kV.

Purification of virus particles

Erythrocytes from infected frogs were suspended in a hypertonic buffer containing 10 mM Tris pH 7.4 and 1 mM EDTA (TE buffer) and lysed using 100 strokes of a Dounce homogenizer. The virus was collected from the erythrocyte lysate by centrifugation at 14,000 *g* for 20 sec and centrifugation of the supernatant at 14,000 *g* for 30 min. The pellet containing virus was suspended in the TE buffer, and centrifuged on a 30% Renografin-76® (Squibb Inc. Canada, Montreal, Quebec, Canada) cushion for 30 min at 90,000 *g*. The virus pellet was resuspended in the TE buffer and repelleted at 14,000 *g* to eliminate any contaminating Renografin-76®.

Structural sensitivities and resistances

A 20 μl drop of the purified virus in TE buffer was incubated at various temperatures for 1 hr. A 5 μl drop of the purified virus in TE buffer was mixed with an equal volume of the test chemical (see Table 1) and incubated at room temperature for 1 min (10 min for ether). The virus samples were placed onto carbon-coated Formvar 150 mesh copper grids and negative contrast stained with 2% phosphotungstic acid. Since FEV could not be propagated in a variety of amphibian, fish and mammalian cell cultures, the viability of the virus after various treatments could not be determined. Instead, the disappearance or marked deformation of the virus indicated structural sensitivity to the temperature or chemical treatment.

Polyacrylamide gel electrophoresis of virus proteins

The procedure followed was similar to that described by Laemmli (1970) as outlined in the Protean I Bio-Rad® (Bio-Rad, Richmond, California 94804, USA) instruction manual. Samples of the purified virus and uninfected erythrocyte lysate (subjected to the same procedure as for the purification of the virus) were boiled in sample buffer containing 6 M urea, glycerol, 2-B-mercaptoethanol, sodium dodecyl sulfate (SDS), and bromophenol blue. The samples were then analyzed on a 10% polyacrylamide gel and stained with silver stain (Wray et al., 1981).

DNA extraction and buoyant density

Purified virions were suspended in TE buffer, 1% SDS and 4% proteinase K (2.5 mg/ml), and incubated at 37 C for 2 hr. The preparation was extracted with phenol, chloroform and isoamyl alcohol. The viral DNA was precipitated with ethanol, resuspended in TE buffer and banded on a CsCl gradient of density 1.690 g/ml. In general, the methods of Maniatis et al. (1982) were followed for these procedures.

Determination of the base composition and thermal denaturation temperature of the viral DNA

The thermal denaturation temperature of the viral DNA was approximated, using the formula developed by Marmur and Doty (1962). The base composition (G + C ratio) was estimated using the formula described by Schildkraut et al. (1962).

RESULTS

Hosts

The prevalence of FEV was highest in the bullfrogs (23.0%). The prevalence in mink and green frogs was 2.3% and 1.8%, respectively. None of the leopard frogs was naturally infected nor could an infection be induced by inoculation with FEV infected blood.

Light and electron microscopy

At the light microscope level, "uninfected" erythrocytes were elliptical, contained a central, ovoid nucleus and measured 14.1 to 16.7 μm (15.3 ± 0.8) by 20.9 to 24.2 μm (23.1 ± 0.9) (n = 500) (Fig. 1). In contrast, "infected" erythrocytes were smaller and more rounded with the nucleus displaced to one end of the cell and the chromatin appearing more condensed. The infected cells measured 12.4 to 15.3 μm (13.7 ± 0.8) by 17.1 to 21.8 μm (19.5 ± 1.1) (n = 500) (Fig. 2). The cytoplasm of infected erythrocytes stained with

Giemsa contained an albuminoid vacuole and a circular red-staining inclusion, which was often surrounded by numerous smaller ones (Fig. 2). Both the large and smaller inclusions fluoresced apple green when stained with acridine orange, and were Feulgen positive, indicating the presence of double stranded DNA. A clear region often surrounded the larger inclusion, creating a "halo" (Fig. 2).

In some bullfrogs up to 90% of the erythrocytes contained large inclusions. At the ultrastructural level, many erythrocytes contained a granular mass, which was interpreted to be the viroplasm or virus factory. Budding from the periphery of the viroplasm were partially assembled cores containing granular material resembling that of the viroplasm (Fig. 3).

The viral core measured 300 to 370 nm from vertex to vertex. The virus particles were observed in three views with five-, three- or two-fold rotational symmetry. Naked and enveloped forms of FEV were observed in negative contrast stained preparations (Fig. 4a, b). No details of the external structure of the core, individual capsomeres and surface projections could be discerned in these specimens (Fig. 4a, b). However, in thin sectioned viruses in situ, outer and inner core membranes which enclosed a central zone containing DNA could be resolved (Fig. 4). Aberrant forms of the virus which had a circular or elongate rather than angular outline were occasionally seen to be budding from a viroplasm.

Stacks of lamellar membranes were associated with, and surrounded, groups of viruses (Figs. 3, 5). The lamellar membranes appeared as two closely apposed membranes separated by an area of the same consistency as the host cell cytoplasm. Extensions of the lamellar membranes surrounded single virus particles and appeared to form an envelope around them (Fig. 4). The diameter of these enveloped forms ranged from 360 to 450 nm.

The viral envelope in negative contrast stained specimens appeared as an electron lucent space surrounding the viral core (Fig. 4a). In thin section, the area between the viral envelope and core was similar to the area between apposing membranes of the lamellar membranes (Figs. 4, 5). The area between the core and the envelope was of the same consistency as the host cell cytoplasm.

Infected erythrocytes often contained a variety of inclusions and a membrane bound, elongate body. The latter occurred in the cytoplasm and often extended the length of the cell (Fig. 6). The inclusion was more dense than the erythrocyte cytoplasm and contained striations arranged parallel to its long axis (Fig. 7). A typical grouping of inclusions commonly seen in the cytoplasm of infected erythrocytes is illustrated in Figure 8.

Structural sensitivities and resistances

The virus was structurally sensitive to the components of cesium chloride and potassium tartrate density gradients. In addition, the virus was sensitive to acidic pH's and exposure at 56 C for as little as 15 min. Lipid solvents such as ether and freon appeared to degrade the viral envelope, leaving only naked cores (Table 1).

Viral proteins

The virus contained at least 16 proteins. The proteins ranged in relative molecular mass (Mr) from 19.5 to 91.0 kilodaltons (kDa). The polypeptides with an Mr of 31 and 43 kDa appeared to comprise a large percentage of the total protein (Table 2, Fig. 9).

Viral DNA

The viral DNA had a buoyant density of 1.690 ± 0.005 g/ml and the residual cell DNA banded at a density of 1.710 ± 0.005 g/ml in a cesium chloride density gradient. The buoyant density of the viral DNA yielded a molar fraction of (G + C ratio) 25 to 36% and a melting temperature of 82 to 86 C.

DISCUSSION

The intraerythrocytic virus in this study, hereafter referred to as frog erythrocytic

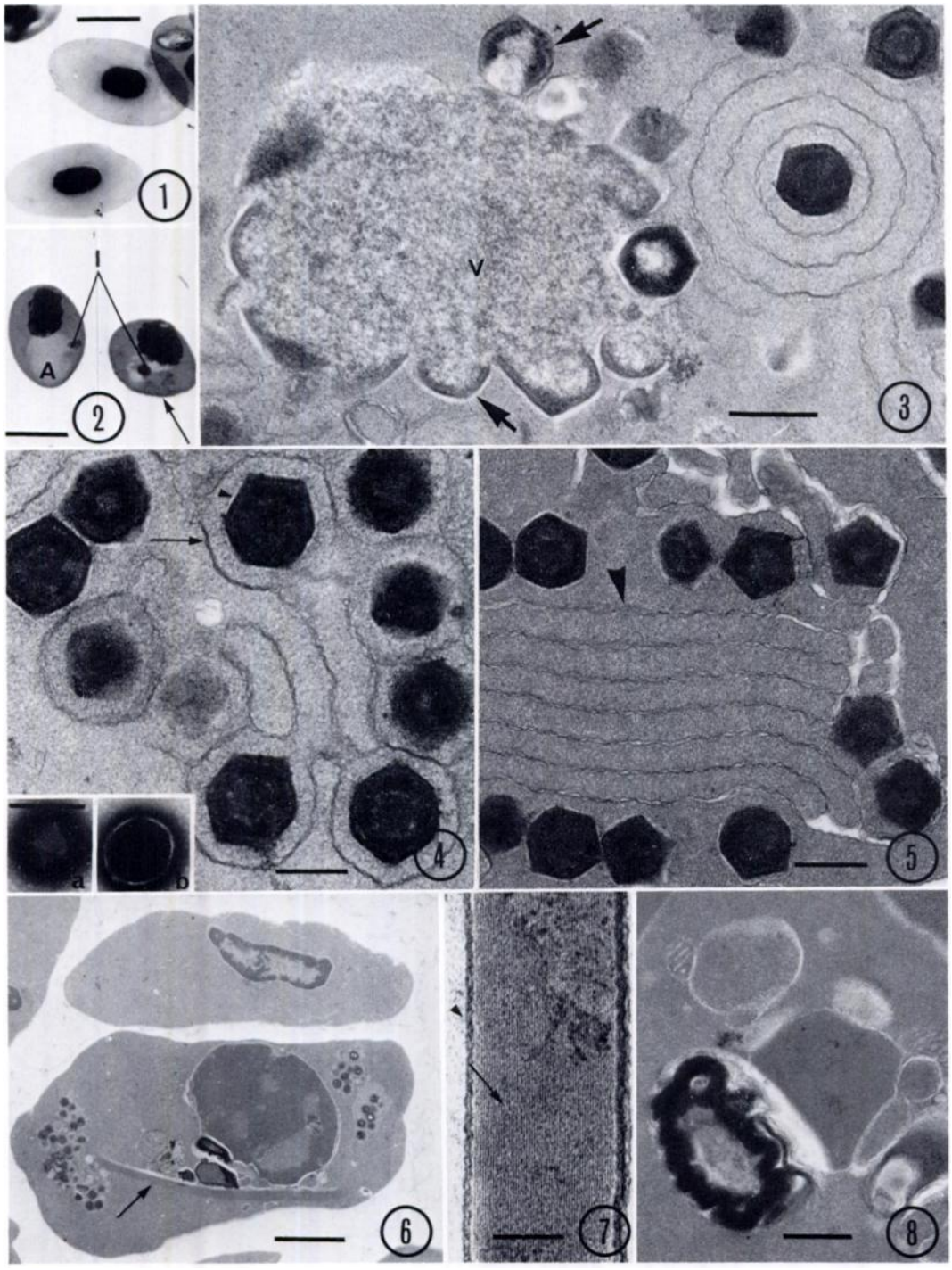

FIGURE 1. Photomicrograph of uninfected frog erythrocytes with typically central nucleus and homogeneous cytoplasm. Giemsa. Bar = 10 μm.

FIGURE 2. Photomicrograph of frog erythrocytes infected with frog erythrocytic virus containing circular cytoplasmic inclusions (I) often surrounded by smaller ones (arrow) and an albuminoid vacuole (A). Giemsa. Bar = 10 μm.

FIGURE 3. A viroplasm (V) and associated virus particles (arrow) seen at the electron microscope level. Note concentric lamellar membranes surrounding a single virion. Bar = 400 nm.

TABLE 1. The structural sensitivity of the frog erythrocytic virus to various reagents and temperatures.

Reagents	Reaction[a]
50% potassium tartrate	sensitive
30% glycerol	sensitive
Cesium chloride (1.45 g/ml)	sensitive
45% Percoll	resistant
100% Renografin-76®	resistant
20% ether	envelope sensitive
50% freon	envelope sensitive
HCl, pH 1	sensitive
HCl, pH 5	sensitive
HCl, pH 7	resistant
HCl, pH 9	resistant
−20 C	resistant
+4 C	resistant
56 C for 15 min	sensitive

[a] Sensitivity was based on the deformation or disappearance of negatively contrast stained virus.

TABLE 2. Number and relative molecular mass of the structural proteins in frog erythrocytic virus.

Number	Relative molecular mass (kDa)
1	91.0
2	85.0
3	70.0
4	69.0
5	63.0
6	62.0
7	59.0
8	54.5
9	51.5
10	45.0
11	43.0
12	37.0
13	34.5
14	31.0
15	29.5
16	19.5

virus (FEV), is the largest intraerythrocytic and icosahedral virus described to date. The FEV shares many features with the *Rana pipiens* intraerythrocytic virus, *Pirhemocyton* spp., *Toddia* spp., tadpole edema virus (TEV), piscine erythrocytic necrosis (PEN) virus, lymphocystis virus, *Nereis* iridescent virus (NIV) and some members of the family Iridoviridae (frog virus 3 [FV 3] and African swine fever virus [ASFV]). These features include an icosahedral core, double stranded DNA, at least 13 virus specific proteins and partial replication in the host cell cytoplasm (Breese and De Boer, 1966; Bernard et al., 1968; Wolf et al., 1968; Wrigley, 1969; Johnston, 1975; Sousa and Weigl, 1976; Devauchelle, 1977; Walker and Sherburne, 1977; Matthews, 1979). FEV appears to exhibit a rather strict host specificity, like some of the above mentioned viruses (Gruia-Gray, 1986).

Many of the inclusions observed at the light microscope level can be related to those seen at the ultrastructural level. It is likely that the large red inclusions at the light microscope level correspond to aggregates of virus particles or the viroplasm. The "halo" seen at the light microscope level probably corresponds to the lamellar membranes surrounding groups of viruses.

FEV has a diameter of 300 to 370 nm and 360 to 450 nm including the envelope. The presence of inner and outer core membranes in FEV is not unusual. Many of the icosahedral viruses possess these two

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FIGURE 4. Frog erythrocytic virus is composed of a lipid envelope (arrow), an icosahedral core (arrow head) and a central dense region containing DNA (D). Extensions of lamellar membranes surround the virus core, resulting in an enveloped virus. Bar = 250 nm.

FIGURE 4a and b. Negatively contrast stained specimens of the frog erythrocytic virus; a naked form (4a) and an enveloped form (4b). Bar = 500 nm.

FIGURE 5. Lamellar membranes (arrow head) are often associated with virus particles. Bar = 300 nm.

FIGURE 6. A membrane bound, elongate body (arrow) often spans the length of an FEV infected erythrocyte. Bar = 3 μm.

FIGURE 7. The membrane bounded (arrow head), elongate body contains striations (arrow) which run parallel to its length. Bar = 200 nm.

FIGURE 8. The cytoplasm of infected erythrocytes often contains a variety of inclusions. Bar = 1 μm.

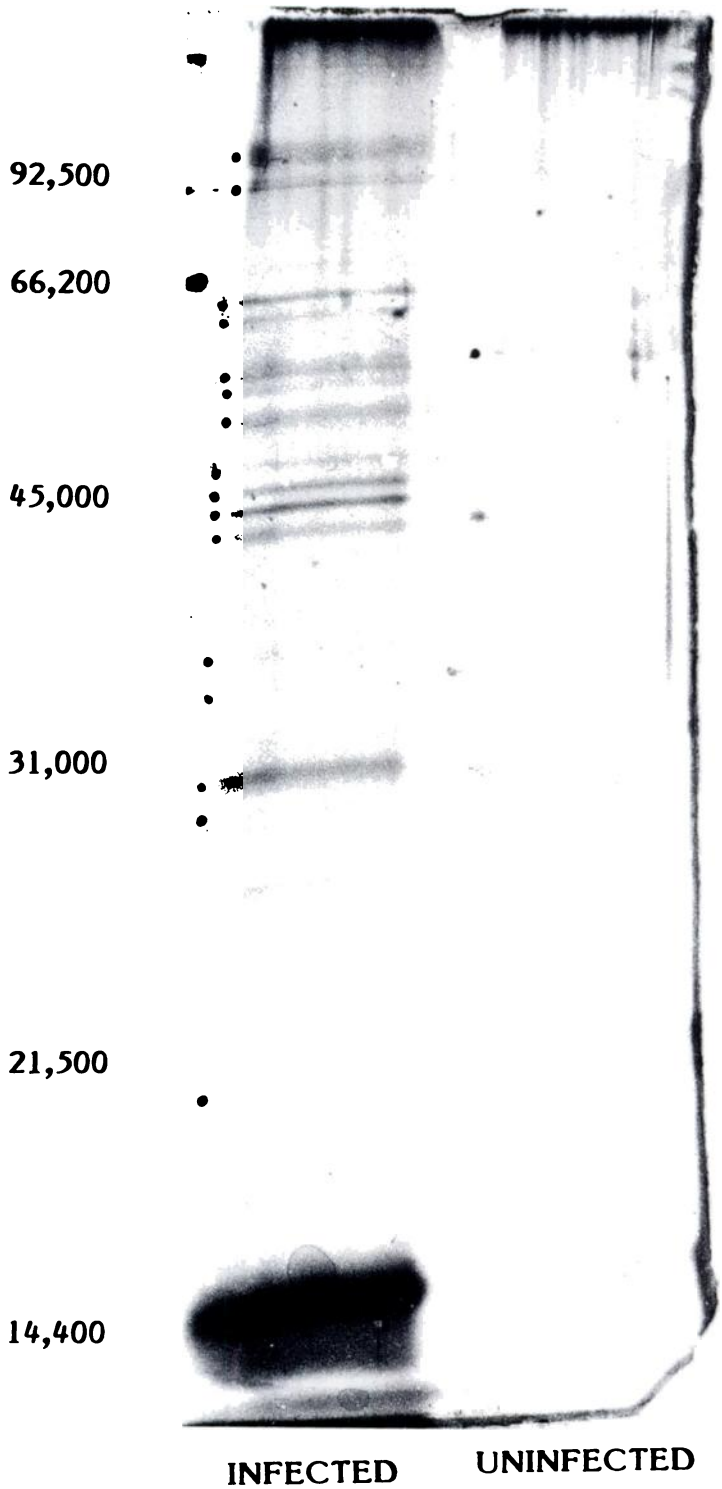

FIGURE 9. At least 16 virus-specific proteins (dots) are observed in a polyacrylamide gel of infected (viral) and uninfected frog erythrocyte lysate.

membranes; the outer is probably responsible for the icosahedral contour of the virus whereas the less stable inner membrane is part of the nucleoid (Stoltz, 1971). Other intraerythrocytic viruses in amphibians which possess these core membranes are the *R. pipiens* virus which measures 200 to 300 nm and 280 to 380 nm enveloped (Bernard et al., 1968) and *Toddia* spp. which measure 170 to 195 nm and 200 to 250 nm including the envelope (Sousa and Weigl, 1976). The aberrant, circular forms of FEV were similar to those seen in NIV (Devauchelle, 1977) and FV 3 (Darlington et al., 1966). The defective particles were scattered among intact icosahedral ones and may have been due to an abnormality in the assembly process.

In keeping with the characteristics of the family Iridoviridae, the viroplasm and FEV contain double stranded DNA. Although DNA synthesis for some DNA viruses occurs in the cytoplasm of the host cell, most likely the viroplasm, it has recently been shown that DNA replication in FV 3 and ASFV occurs initially in the nucleus (Goorha et al., 1978; Tabarés et al., 1980; Devauchelle et al. 1985). Devauchelle et al. (1985) suggested that as the icosahedral core is formed, it "sequesters" a portion of the viroplasm, eventually resulting in a complete nucleocapsid. Evidence for this type of developmental sequence for FEV can be seen in Figure 3. However, there was no evidence to suggest how the virus enters and exits the erythrocytes, nor whether the nucleus plays a role in the replication of FEV.

Not all the intraerythrocytic viruses or the members of the Iridoviridae possess an envelope. Among those viruses that are enveloped, such as ASFV and FV 3, the envelope is acquired either by budding through the host cell membrane or de novo in the cell cytoplasm (Willis and Granoff, 1974; Carrascosa et al., 1984). There is no evidence that the FEV acquires its envelope by these conventional methods. Data from this study indicate that the viral envelope originates from lamellar membranes surrounding the virus particles, since the area between the envelope and viral core is similar to the area between the apposing membranes of the lamellar membranes. Similar lamellar membranes were described by Bernard et al. (1968) in virus-infected erythrocytes from *R. pipiens*. They suggested that the lamellar membranes may have been residual membranes of the endoplasmic reticulum or that the virus induced the erythrocyte cytoplasm to produce the membranes. However, they did not show any association between the viral envelope and the lamellar membranes. A similar type of membrane configuration was found in erythrocytes infected with *Toddia* spp. Groups of *Toddia* spp. were often surrounded by a single membrane (Sousa and Weigl, 1976). As viral (FEV) inclusions were found primarily in the cytoplasm of mature erythrocytes, which generally lacks membranes, it is likely that the lamellar membranes associated with the virus particles are formed in response to infection of the host cell by the virus.

The elongate striated structures often observed in infected erythrocytes appeared similar to the tubular forms seen in cells infected with NIV (Devauchelle, 1977), FV 3 (Darlington et al., 1966; Kelly and Atkinson, 1975) and *Toddia* spp. (from Sousa and Weigl, 1976). However, unlike NIV and FV 3, the elongate forms in FEV infections were never found to terminate in structures resembling a viral core, and were therefore not considered to be aberrant forms of FEV. Unlike FEV infected erythrocytes, in which the elongate striated bodies occurred exclusively in the cytoplasm, Sousa and Weigl (1976) observed similar bodies in the nucleus and cytoplasm of cells infected with *Toddia* spp. They claimed that the crystalline structure of these inclusions resembled the polyhedron associated with the polyhedrosis viruses, the capsules of the granulosis viruses as well as the inclusion bodies associated

with the entomopox viruses. Sousa and Weigl (1976) believed that these structures were composed of excess core protein. It is likewise possible that the elongate striated bodies associated with FEV are composed of residual core protein or that they might be a condensed form of hemoglobin (Fawcett, 1969). The fine striations observed within these inclusions are reminiscent of a paracrystalline form of deoxygenated hemoglobin found in uninfected cod erythrocytes (Thomas, 1971). The clusters of smaller vesicular inclusions associated with FEV are similar to ones observed in PEN infected erythrocytes of Atlantic herring (Reno et al., 1978). The vesicular inclusions are less electron dense than the cytoplasm and are surrounded by multiple membranes. The origin and nature of the cytoplasmic inclusions associated with FEV and PEN infections are not known, but it is likely that these inclusions are cytopathological alterations of infected erythrocytes.

Unlike many viruses including the iridoviruses, FEV appears structurally sensitive to changes in the osmotic concentration, and consequently was difficult to purify. FEV's sensitivity to osmotic changes may be due to its large size. The physiochemical properties of FEV vary slightly from that of the members of the family Iridoviridae. Similar to other enveloped iridoviruses (FV 3 and ASFV) FEV is sensitive to lipid solvents. FEV appears structurally stable at pH 7 to 9, whereas the iridoviruses are stable at pH 3 to 10. Both FEV and the iridoviruses are stable at −20 to 4 C, but sensitive to at least 15 min treatment at 56 C (Matthews, 1979).

The relative molecular masses of the 16 proteins of FEV range from 19.5 to 91.0 kDa, with two major viral proteins. These proteins fall within the same range as those of FV 3 (17 structural proteins, with an Mr from 8.5 to 100.0 kDa, and one major viral protein) (Tan and McAuslan, 1971; Goorha and Granoff, 1974) and ASFV (28 structural polypeptides ranging from 11.5 to 243 kDa, and six major viral proteins) (Tabarés et al., 1980).

The buoyant density and G + C content of the double stranded DNA of FEV (1.690 ± 0.005 g/ml; 25 to 36%) are similar to those of the iridescent viruses (1.689 to 1.702 g/ml; 29 to 39%) (Bellet and Inman, 1967; Kelly and Avery, 1974) and ASFV (1.700 ± 0.003 g/ml; 41%) (Enjuanes et al., 1976). Experiments in progress will determine whether the molecular mass and restriction sites of the DNA of FEV are also similar to those of the iridescent viruses and ASFV.

Based on the similarities in morphology and biochemical properties between FEV and the iridoviruses, FEV is a suitable candidate member of the family Iridoviridae. However, to include FEV, the size of members of this family (which ranges from 125 to 300 nm; Matthews, 1979) would have to be extended to 450 nm. To be more certain of the classification of FEV further characterization of the DNA (in progress) is necessary.

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