



Partial characterization of an entomopoxvirus isolated from grasshoppers  
by Herbert Carl Kussman

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE  
in MICROBIOLOGY

Montana State University

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Abstract:

Arphia poxvirus (APV), an entomopoxvirus originally isolated from *Arphia conspersa*, is also infective in *Camnula pellucida* and *Xanthippus corallipes*. Infection occurs in fat body of the host causing premature death. Inclusions appear oval to ellipsoid and, when mature, measure approximately 11-13  $\mu\text{m}$  in diameter. Virions are brick-shaped, about 300 nm in width by 400 nm in length, and contain a large double-stranded DNA core surrounded by a triple-layered intimal membrane. The *Arphia* poxvirus was found to be distinct from two other entomopoxvirus from grasshoppers, the grasshopper inclusion body virus (GIBV) and *Phoetaliotes* poxvirus (PPV), as determined by differences in host range electrophoretic patterns of viral proteins, and antigen-antibody gel diffusion reactions.

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March 1, 1978

PARTIAL CHARACTERIZATION OF AN ENTOMOPOXVIRUS  
ISOLATED FROM GRASSHOPPERS

by

HERBERT CARL KUSSMAN, JR.

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of the requirements for the degree

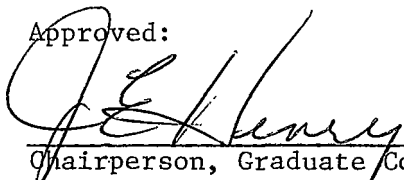
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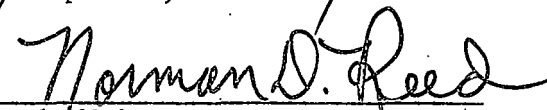
MASTER OF SCIENCE

in

MICROBIOLOGY

Approved:

  
Chairperson, Graduate Committee

  
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MONTANA STATE UNIVERSITY  
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## ABSTRACT

Arphia poxvirus (APV), an entomopoxvirus originally isolated from Arphia conspersa, is also infective in Camnula pellucida and Xanthippus corallipes. Infection occurs in fat body of the host causing premature death. Inclusions appear oval to ellipsoid and, when mature, measure approximately 11-13  $\mu$ m in diameter. Virions are brick-shaped, about 300 nm in width by 400 nm in length, and contain a large double-stranded DNA core surrounded by a triple-layered intimal membrane. The Arphia poxvirus was found to be distinct from two other entomopoxvirus from grasshoppers, the grasshopper inclusion body virus (GIBV) and Phoetaliotes poxvirus (PPV), as determined by differences in host range, electrophoretic patterns of viral proteins, and antigen-antibody gel diffusion reactions.

## Chapter 1

### INTRODUCTION

According to Bellet et al. (2), there are three major groups of inclusion viruses that cause disease in insects: the baculoviruses, which consist of the nuclear polyhedrosis viruses and the granulosis viruses; the cytoplasmic polyhedrosis viruses; and the entomopoxviruses. Most of the early studies of viral diseases in insects have centered on the nuclear polyhedrosis viruses and the cytoplasmic polyhedrosis viruses. Baculoviruses contain deoxyribonucleic acid (DNA) as genetic material and replication occurs in the nucleus of the host cell. Entomopoxviruses also contain DNA, but replication occurs in the cytoplasm of infected cells. The genetic material of cytoplasmic polyhedrosis virus is ribonucleic acid (RNA) and this virus replicates in the cytoplasm.

Hurpin and Vago (13) isolated the first entomopoxvirus. The original characterizations of this and other entomopoxviruses were based on electron microscopic examinations (3, 10, 24, 26) that provided information on the structure of the inclusion bodies with associated protein matrices, information on the structure of the occluded virions and insight into the development of virions and inclusions (6, 8, 10, 13, 26). More recent investigations established the techniques necessary for the extraction of the virions from the inclusion matrix

in order to determine the chemistry and structure of the virions (1, 16, 19, 25). These studies have established that the virions of entomopoxviruses are brick-shaped, each possessing a central core of DNA surrounded by a limiting membrane consisting of three distinct layers (6, 9). The DNA is a large, complex molecule containing the genes responsible for the production of at least four enzymes involved in replication (23, 19). As additional examples of the general characteristics of entomopoxviruses, Landridge and Roberts (16) have shown by electron microscopy that the DNA molecule of Amsacta poxvirus was 64.5  $\mu$ m long and Arif (1) established that the bouyant density of the DNA of the Choristoneura entomopoxvirus was 1.6838 gm/ml. As pointed out by Bergoin et al. (3) and McCarthy et al. (20), the entomopoxviruses of insect and the vertebrate poxviruses exhibit many similar characteristics.

The above studies were performed on entomopoxviruses isolated from Lepidoptera (1, 9, 19, 25), Coleoptera (6, 13), and Diptera (4, 7, 26). The first entomopoxvirus reported from grasshoppers (Acrididae: Orthoptera) was isolated from Melanoplus sanguinipes by Henry and Jutila (11), at which time they suggested it to be a nuclear polyhedrosis virus. More recently, Henry et al. (12) established that this virus has the characteristics of an entomopoxvirus, and referred to it as the grasshopper inclusion body virus (GIBV). GIBV has been

subjected to electron microscopic studies, and Landridge and Roberts (15) have reported that the DNA is 62.8  $\mu$  long with a molecular wt. of  $123.2 \times 10^6$  daltons. Two similar entomopoxviruses have been isolated from different species of grasshoppers; one from Arphia conspersa has been labelled as the Arphia poxvirus (APV), and a second from Phoetaliotes nebrascensis called the Phoetaliotes poxvirus (PPV). Except for some preliminary electron microscope work, comparative studies have not been completed.

#### Statement of Objectives

The purposes of this study were:

- (1) To further characterize, both biochemically and biophysically, the entomopoxvirus (APV) isolated from A. conspersa.
- (2) To determine morphological and chemical relationships between APV and the other entomopoxviruses, GIBV and PPV, isolated from grasshoppers.

## Chapter 2

### MATERIALS AND METHODS

#### Experimental Inoculations

The two methods used to inoculate grasshoppers were per os and injection. For per os inoculations, grasshoppers were fed directly a 5  $\mu$ l drop of a viral suspension that was placed on the mouth parts of third-instar nymphs. Also grasshoppers were inoculated indirectly by placing a 5  $\mu$ l drop of a viral suspension on a small lettuce disc (5.0 mm diameter). The drop was allowed to dry and the disc was fed to a third instar grasshopper. Grasshoppers were inoculated by injecting approximately 5  $\mu$ l of a viral suspension using a 0.25 tuberculin syringe with a 27 gauge needle. Injections were made intrathoracically into third-instar grasshoppers. Viral concentrations between  $10^1$  to  $10^3$  inclusions per 5  $\mu$ l were used for all inoculations.

#### Isolation of Virus from Grasshoppers

Preliminary diagnosis of viral infection was conducted by homogenizing grasshoppers in Thomas tissue homogenizers equipped with teflon pestles and examining the homogenates with a light microscope using phase contrast optics. Homogenates that contained viral inclusions were pooled, passed through two layers of cheesecloth or gauze, and placed in 50 ml centrifuge tubes and centrifuged at 2,500 x g for 15 min. The sediment was washed, repelleted (2x), and stored

in distilled water at  $-10^{\circ}\text{C}$  until needed.

Purification of Viral Inclusion Bodies by Sucrose Density Gradient  
Centrifugation (SDG)

Discontinuous sucrose gradients of 60, 55, 50, 45, 40% (weight/volume) were prepared in 16 ml cullulose nitrate centrifuge tubes. Viral preparations from grasshopper tissues were layered onto the top of the gradients and were centrifuged ( $64,680 \times g$ ) for 2 h at  $-5^{\circ}\text{C}$  in a Beckman L3-40 preparative ultracentrifuge using a SW 27.1 swinging bucket rotor. After centrifugation, the band containing the inclusions was removed using a Pasteur pipette and a propipette and was washed twice with distilled water. This entire centrifugation process was repeated 3 times.

Breakdown of Viral Inclusion Bodies

Density gradient purified preparations containing  $10^{10} - 10^{12}$  viral inclusions were pelleted and resuspended in about 5 ml of a solution of 0.1M sodium carbonate and 0.1M sodium thioglycolate in distilled water, adjusted with 0.3N sodium hydroxide to a pH of 11.5. The preparation was incubated overnight at  $27^{\circ}\text{C}$ , after which time the preparations were placed onto preformed linear sucrose gradients (55, 45, 35, 25, 20% w/v) and centrifuged at  $64,680 \times g$  for 4 h. At the end of this time, the virions were collected from the sucrose

tubes by inserting a 22 gauge needle, with a 10 cc syringe attached, through the tube and drawing off the virions. The virions were then pelleted at 64,680 x *g* for 30 min. and resuspended in sterile distilled water. Penicillin (1,000 units/tube) was then added, and the preparations were frozen until needed.

#### Preparations of Materials for Electron Microscopic Examination

Grasshopper fat tissues were fixed in 4% glutaraldehyde in phosphate buffer for 24 h at 4°C, washed in buffer for 14 min., then postfixed in osmium tetroxide (2%) for 2 h at 4°C. Tissues were dehydrated through a graded acetone series (50, 70, 85, 95, 100%), placed in two changes of propylene oxide (100%), placed in two solutions of propylene oxide-epon araldite in a ratio of 2:1 and then 1:1, and embedded in epon araldite. Tissue blocks were hardened in a vacuum oven for 51 h and sectioned on a Reichert OM U2 ultramicrotome. Sections were stained with aqueous uranyl acetate for 3 min. and Reynold's lead citrate for 1 min.

For electron microscopic examination, the virions freed from inclusions by the above method were placed on formvar coated grids and were either negatively stained with 2% phosphotungstic acid for direct examination or shadow casted with platinum at a 3:1 (length to height) ratio for indirect determination of size and structure. All examinations were made with a Zeiss EM-9 microscope.

Photomicrographs from the electron microscope were made on Kodak 4489 Electron Microscope film.

Determination of Total Protein of Inclusions and Virions

Suspensions of  $6.56 \times 10^6$ ,  $3.05 \times 10^6$ ,  $1.53 \times 10^6$ , and  $7.55 \times 10^5$  inclusions per ml were prepared and checked by direct counts using a Neubauer hemacytometer. One ml of each preparation was centrifuged, the water decanted, and the pellet dissolved in 0.5 ml of 1N NaOH overnight in cellulose nitrate tubes. Protein determinations were then carried out using a modification of the Lowry method (14, 21). Standard curves were obtained using 13 concentrations of bovine serum albumin, ranging from 600  $\mu\text{g}$  per 0.5 ml to 5  $\mu\text{g}$  per 0.5 ml, and a blank prepared in 1N NaOH. These were prepared by first dissolving crystallized bovine serum albumin in a small amount of distilled water and adjusting to volume with 1N NaOH. Ten absorbance readings were made for each dilution of the standard and these were averaged and plotted on normal graph paper. The logs of both concentration and absorbance reading averages were also determined and plotted on 2 X 2 cycle log graph paper. These results were used as linear correlation models, from which an equation was derived using a Wang calculator, thus allowing determination of the protein concentrations of inclusions without graphing error. In the established equation  $X = (\text{Log } Y - 4.352624) / .73098732$ , X equals the protein concentration and Y is the absorbance

reading. Eleven absorbance readings were obtained for each viral inclusion concentration from which the highest and lowest were deleted and the average was based on the remaining 9 readings. Using these averages as the values of Y, the protein concentrations (X) are then determined, after which the total protein per inclusion sample was assessed according to a  $10^3$  inclusion baseline. Total protein in preparations of virions released from similar concentrations of inclusions by the method reported above also were determined by the modified Lowry technique. The estimate of total protein in inclusion preparations and in viral preparations were compared to estimate percentage of the total protein in inclusions and in virions.

#### Electrophoresis of Proteins

Viral inclusions, purified by density gradient centrifugation, were suspended in 0.1M sodium carbonate and 0.1M sodium thioglycolate (pH 11.5) overnight at 25°C, after centrifugation at 2,500 x g for 5 min. to remove undissolved inclusions and fragments, the supernatant was decanted and precipitated by the addition of 2 to 3 volumes of 10% trichloroacetic acid. The precipitate was pelleted by centrifugation at 900 x g for 5 min., the clear supernatant decanted, and washed once with 5% trichloroacetic acid and twice with 100% acetone. The precipitate was then dissolved in a mixture of 3 ml of 1% sodium dodecyl sulphate (SDS), 3 ml spacer gell buffer (1:8 dilution), 0.1 ml

of 2-mercaptoethanol, and 2 ml of 10  $\mu$ l/l bromphenol purple solution. The solution was brought to 10% with respect to sucrose (40%) and heated to 100°C in a water bath until the precipitate dissolved completely.

Some viral inclusions were placed into 10% SDS overnight. Sucrose was added to a final concentration of 10% along with bromphenol blue as a marker (18). The solutions were layered into troughs cut in 5% polyacrylamide gel as a spacer gel and electrophoresed through 13% polyacrylamide gel at 25 milliamps for 4 h. The gels were stained in 25% Coomassie blue in 9% acetic acid, 50% methanol for 2 h, destained in 9% acetic acid in 5% methanol overnight, and examined by locating the blue staining areas. Gels were stored in 7.5% acetic acid in 5% methanol.

#### Preparation and Collection of Immune Sera

Sera containing antibodies to Arphia poxvirus inclusions (APV-I), grasshopper inclusion body virus inclusions (GIBV-I) or to virions extracted from APV inclusions were obtained from rabbits after 4 weekly inoculations according to the following regimen:

First week - Inclusions or virions with Freund's complete adjuvant (Colorado Serum Co.) - 4 ml

Second week - Inclusions or virions with Freund's incomplete adjuvant (Grand Island Biological Co.,) - 4 ml

Third week - Inclusions or virions with Freund's incomplete adjuvant - 2 ml

Fourth week - Inclusions or virions with Freund's incomplete adjuvant - 2 ml

Injections were given intradermally above the axillary lymph nodes, using a 22 gauge needle. Five days after the final injection 50 ml of blood were withdrawn. The blood was transferred to 40 ml plastic centrifuge tubes and after 1 h at 22°C (room temperature) the clot was released from the sides of the tubes with a metal rod and the tubes were incubated overnight at 4°C. The serum was drawn off with a syringe, transferred to serum bottles and placed in storage at -10°C.

#### Preparation of Ouchterlony Plates

Antigenic relatedness among the viruses was assessed by the double-diffusion in two dimensions (Ouchterlony) method (22). Four ml of sodium barbitol buffer (1.03% w/v sodium barbitol plus 1.25% v/v 1N HCl in distilled water) containing 1% purified agar (Difco Corp.) and 0.1% sodium azide were added to 60 X 15 mm petri dishes (Falcon plastics). Six peripheral wells, evenly spaced, were located 8 mm from the edge of a central well. Antigen and serum were added to the wells and the plates were incubated 4 days at 4°C, after which they were washed overnight in distilled water and stained with a solution of 0.1% Coomassie blue dye in acetic acid:ethanol:distilled

water (1.0:4.5:4.5) for 2 h, then destained with a solution of acetic acid:ethanol:distilled water (1.0:2.5:6.5) for 4 h. Reaction zones were located using an indirect light box.

Photographs of plates were made using a 35 mm camera, with bellows attachment, and a Vivitar No. 25A red filter on Kodak Panatomic X black and white film (ASA 32). Indirect lighting was applied from beneath.

#### Feulgen Reaction for Nucleic Acid Type Determination

Nucleic acid type, deoxyribonucleic acid or ribonucleic acid, was determined by use of the Feulgen reaction. Purified inclusions were fixed in Arnoy's fixative, hydrated, incubated in 1N HCl, stained in Schiff's Reagent (0.01% basic fuchsin w/v, 0.02%  $\text{Na}_2\text{HOP}_4$  w/v, 0.2% 1N HCl, v/v in distilled water, counterstained in fast green, mounted, and examined (21). Viral inclusions containing DNA stain purplish-red by this technique. RNA containing virions are not stained by this technique.

#### Acridine Orange Staining to Determine Strandedness of DNA

Virions extracted from APV inclusions were stained with the fluorochrome acridine orange to establish if the DNA was double- or single-stranded. Purified virions were dried on a cover slip, fixed in Carnoy's fixative, hydrated, rinsed in McIlvaine's buffer (pH 4.0),

stained in 0.1% acridine orange in McIlvaine's buffer, mounted and examined with a Zeiss fluorescent microscope (17). Double-stranded DNA viruses fluoresce yellow-green by this technique. Barley stripe mosaic virus (BSMV), a single-stranded RNA virus, was stained as a control. Single-stranded viruses fluoresce red by the above technique.

#### Extraction of DNA from Virions

Deoxyribonucleic acid was extracted from the APV virions by the method of Gafford and Randall (5). Saline citrate buffer with EDTA and sodium lauryl sulfate in 95% ethanol were added to the virion suspension and the solution was carefully mixed. After 45 min. the solution was mixed with glycerol-bromphenol marker and electrophoresed through 0.7% agarose gel tubes, 12 cm long, on a Bio-Rad disc-gel electrophoresis instrument at 4 milliamps per tube for 3 h. Tubes were then stained with ethidium bromide and examined under UV light. Nucleic acid fluoresces yellow-orange with this technique.

## Chapter 3

### RESULTS

#### Gross Pathology of Virus Infections

Arphia poxvirus (APV), was originally isolated from Arphia con-  
spersa collected from the Spanish Creek drainage of Gallatin County,  
Montana. It has been experimentally transmitted to other Arphia con-  
spersa and to Camnula pellucida and Xanthippus corallipes, all of which  
are banded-wing grasshoppers of the subfamily Oedipodinae. These  
experimental inoculations were per os using preparations containing  
either occluded (virions in inclusions) or nonoccluded (virions  
removed from inclusions) virions and by intrahemocoelic inoculations  
of preparations of purified nonoccluded virions. Melanoplus sanguinipes,  
M. bivittatus, Schistocerca americana, and Schistocerca vaga vaga,  
which belong to the traditional spur-throat grasshopper subfamily of  
Cyrtacanthacridinae, and Aulocara elliotti, of the subfamily Acridinae,  
were refractory to infection when inoculated either per os or by  
intrahemocoelic injection in that disease symptoms were not expressed  
by inoculated grasshoppers nor were inclusions evident when tissues  
were examined.

In the terminal phases of infection by APV, grasshoppers appeared  
sluggish and pale in color. Their abdomens were distended and they  
exhibited signs of ataxia. When inoculated per os as third instar

nymphs, development was slowed during the fourth or fifth nymphal instars and they often died prior to development to the adult stage. If inoculated per os with a high concentration of virus or if inoculated by injection, the nymphs often failed to develop beyond the third instar stage during which they either died or persisted in a moribund state for prolonged periods. Inclusions were detectable in susceptible tissues at approximately 14 days after per os inoculation, but gross signs of infection, particularly ataxia, were evident at about 10 days post-inoculation.

#### Histopathology and Viral Replication

Infection generally was restricted to the fat tissues of the grasshoppers which, upon gross dissection of infected cadavers, appeared hypertrophied and nearly filled the body cavity. The fat tissue became increasingly pale yellow in color and granular textured as opposed to the normal shiny-yellow, smooth characteristic.

Examination of thin sections of fat tissue by electron microscopy revealed that replication occurs within the cytoplasm of cells (Figure 1). The earliest stages of viral replication observed were virogenic stroma (Figure 2) that appeared as electron dense amorphous masses located within the cytoplasm at 10 days post-inoculation. These stroma contained both coarse and fine granular material that possibly represented pools of protein and DNA. Immature virions were observed

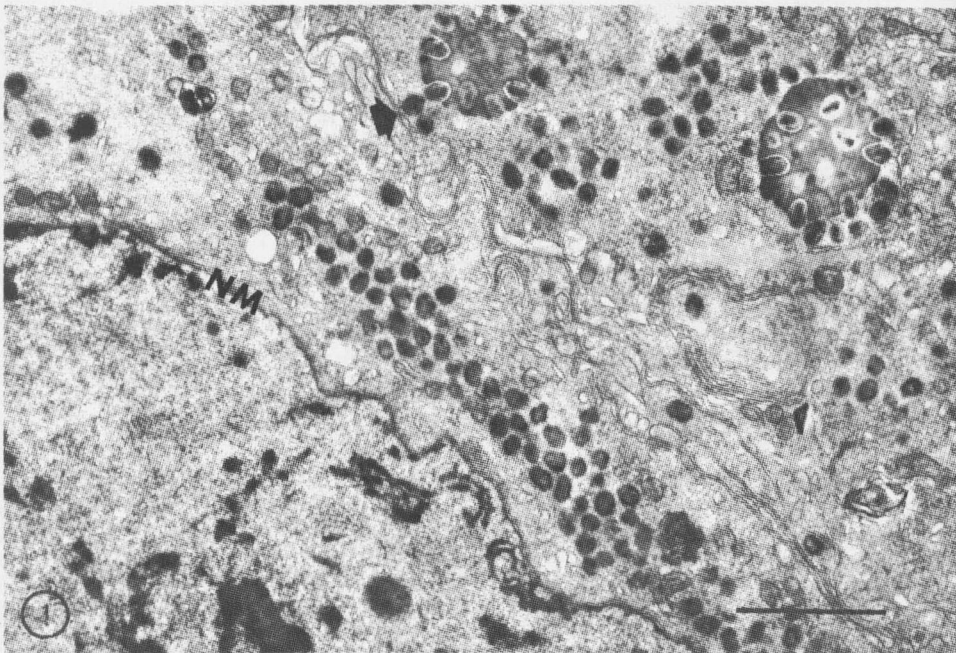


Figure 1. Electron micrograph showing nonoccluded virions of APV aligned along the nuclear membrane (NM) in the cytoplasm of an infected fat cell. Adjacent cell (arrow points out cell membrane) is in a more advanced stage of infection as indicated by the development of inclusions.  
Line Scale = 2.5  $\mu$

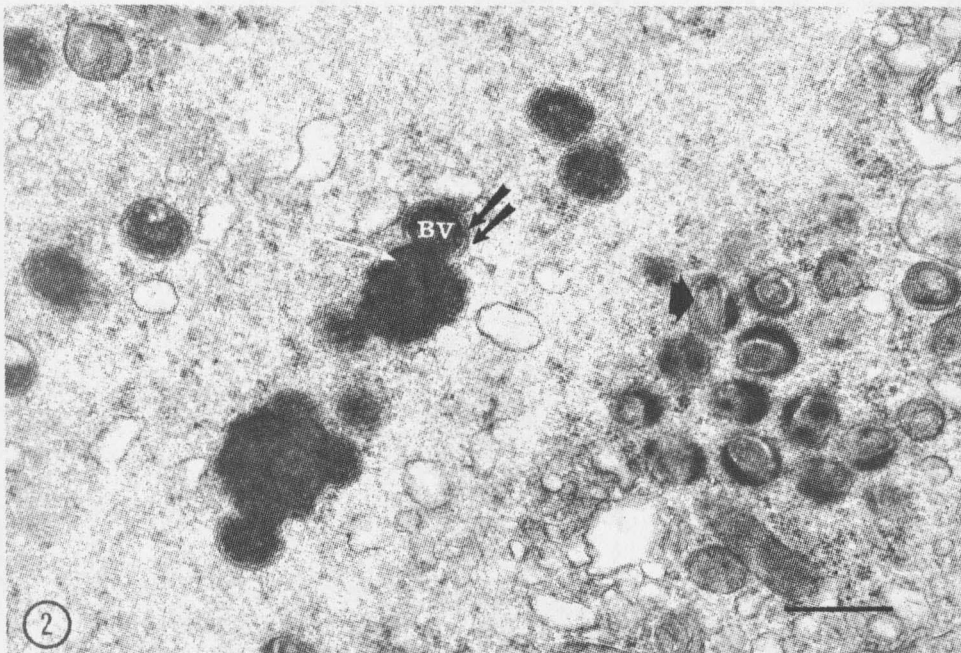


Figure 2. Electron micrograph of virogenic stroma with fine (arrow) and coarse granular areas. The trilaminar membrane (double arrow) surrounds the virion "budding" from the stroma. Other virions free in the cytoplasm appear in a later developmental form (heavy arrow).  
Line Scale =  $0.5 \mu$

budding from the stroma and appeared surrounded by a trilaminar encapsulating membrane. At this early stage, the virions appeared oval with an amorphous, granular internal structure. Other virions, presumed to be older, possessed a definite elongated form with the internal area differentiated into protein and nucleic acid core components. Initial inclusion body formation usually occurred in areas where virions were abundant (Figure 3). A lighter halo was seen surrounding each virion as it was occluded. Maturing inclusions appeared increasingly electron dense apparently due to the increased concentration of virions. During occlusion, most virions on the surface of the developing inclusion were oriented such that the longitudinal axis was perpendicular to surface of the inclusion (Figures 4, 5). Mature inclusions possessed a peripheral region that was devoid of virions (Figure 6).

The effects of infection were most evident in cells that contained numerous inclusions. Lipid granules, readily apparent at the start of infection, were not evident in later stages. Normal cellular constituents, including mitochondria and Golgi bodies, were displaced and the endoplasmic reticulum appeared distended (Figures 3, 7). The fat bodies from grasshoppers in the terminal stage of infection lacked normal cytoplasmic structure but were virtually filled with inclusions that caused expansion of the fat tissue. Resulting death was considered

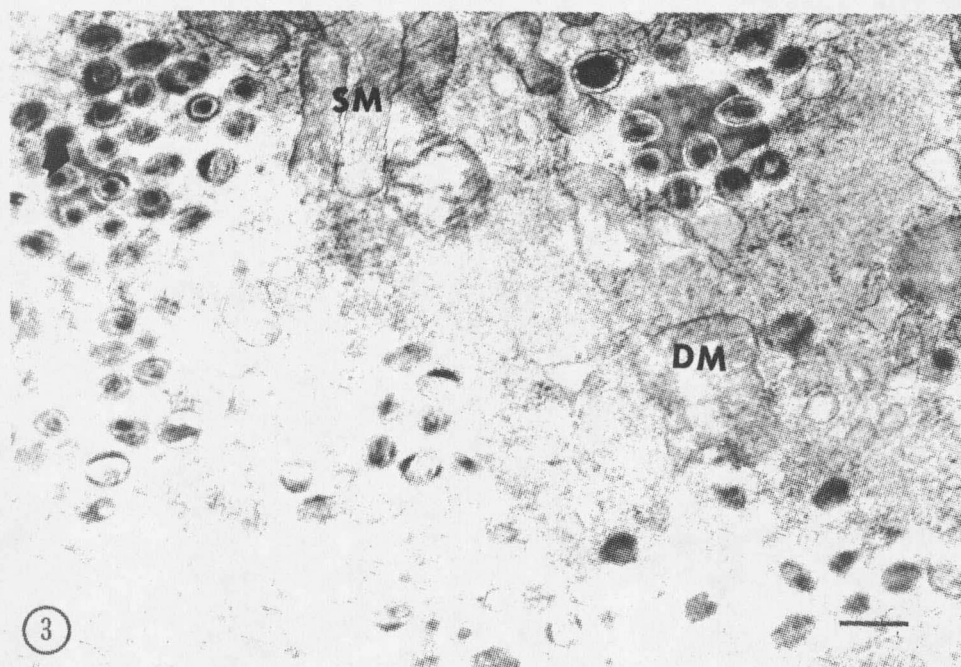


Figure 3. Electron micrograph showing an inclusion in an early developmental stage (arrow). Some mitochondria appear distended (DM) or displaced (SM), a condition characteristic of APV infections.  
Line Scale = 0.5  $\mu$

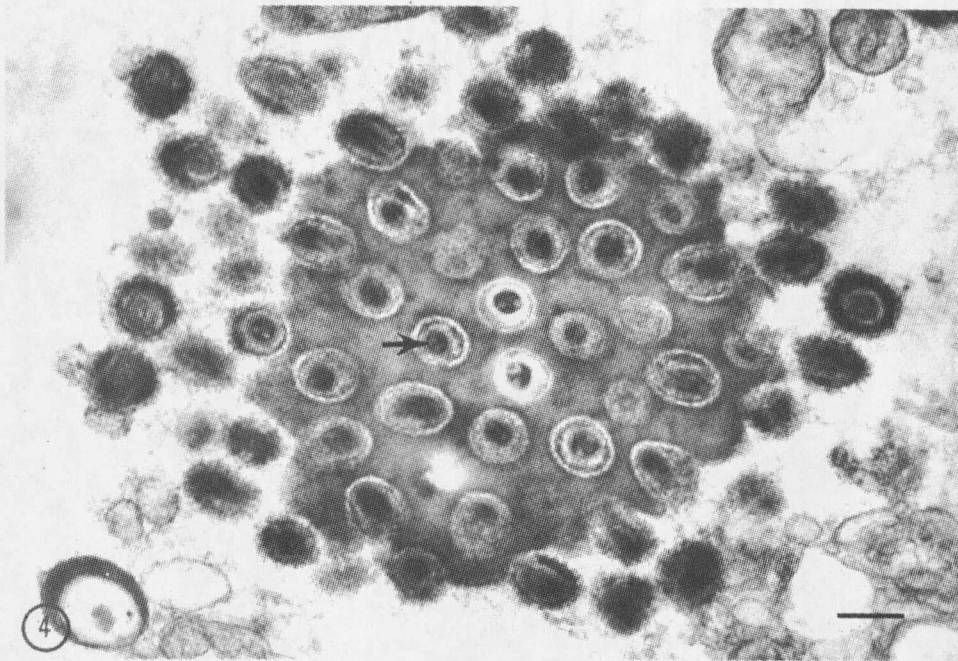


Figure 4. Electron micrograph of an inclusion showing the internal structure of occluded virions, particularly the electron dense core with less dense DNA cores.  
Line Scale =  $-.3 \mu$

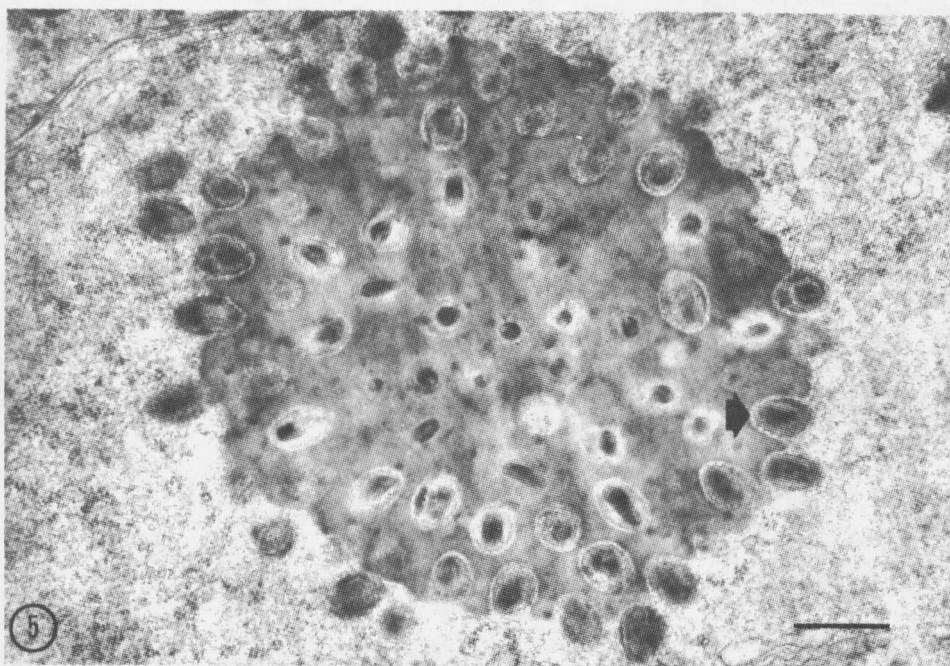


Figure 5. Electron micrograph of an inclusion body in a late stage of development showing the orientation of virions on the inclusion surface and the halo and capsid with spherical subunits around the virions (arrow).  
Line Scale -  $0.5 \mu$

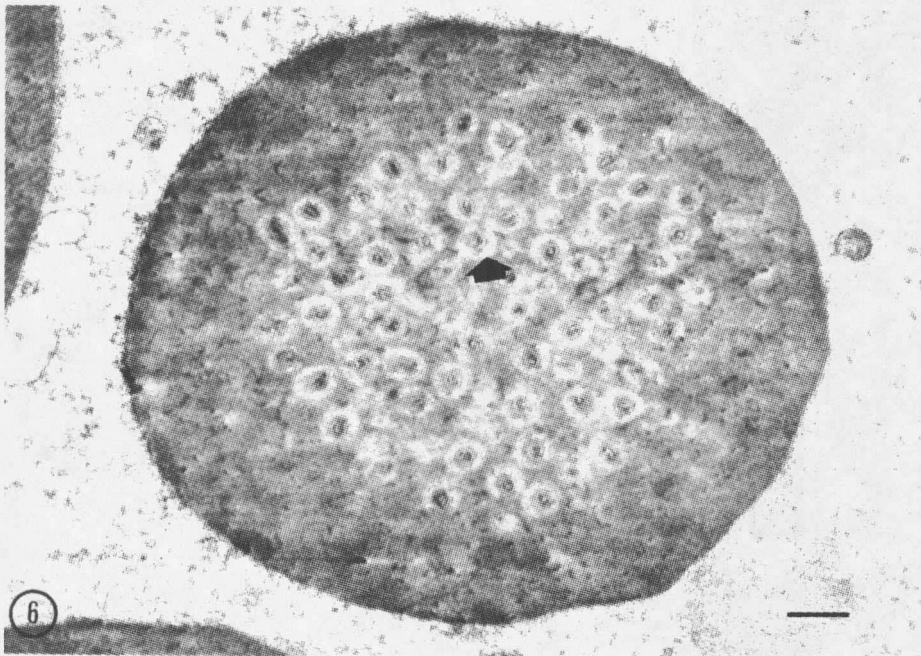


Figure 6. Electron micrograph of a mature inclusion with occluded virions concentrated in the central area of the inclusion (arrow) and an avirogenic peripheral area.  
Line Scale -  $0.6 \mu$

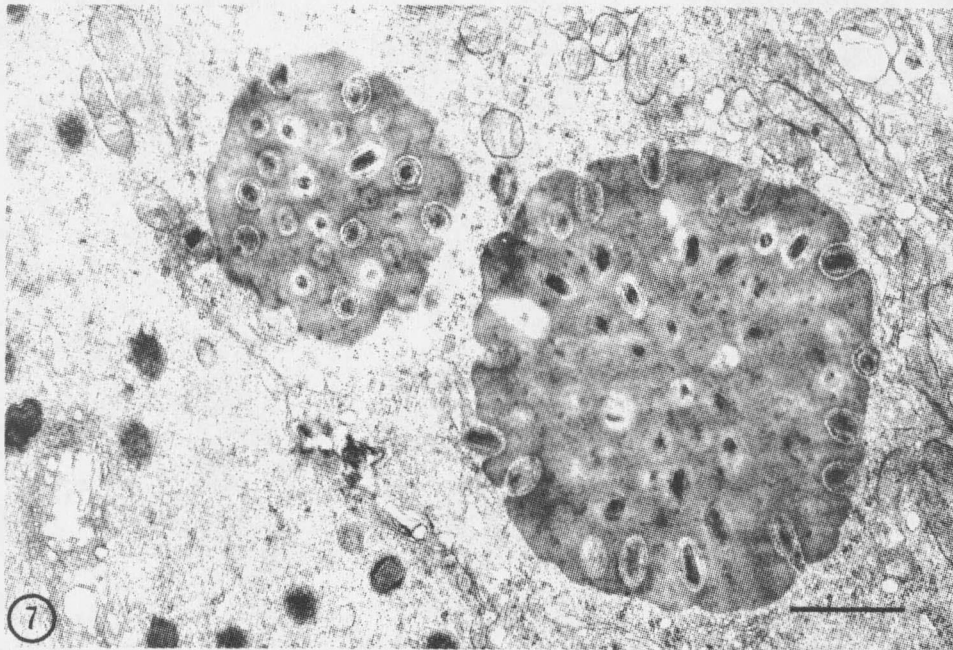


Figure 7. Electron micrograph of two inclusion bodies in early developmental stages. Note the displacement of mitochondria and general lack of endoplasmic reticulum. Line Scale = 1.0  $\mu$

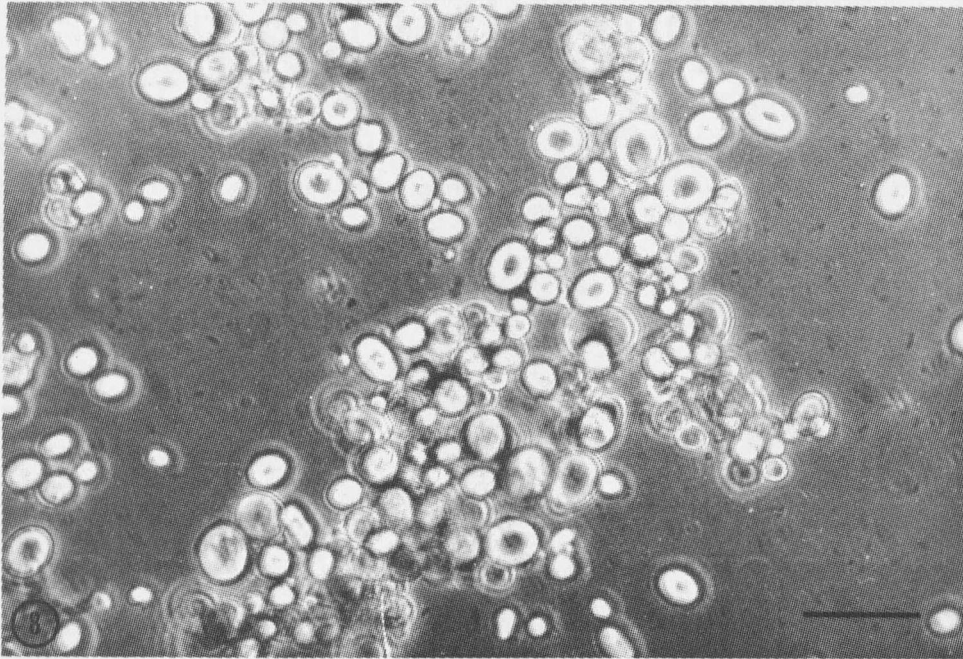


Figure 8. A fresh preparation of purified inclusions of APV under the light microscope using phase contrast optics.  
Line Scale - 30.0  $\mu$

due to deprivation of the energy reserves required by the host for growth.

#### Physiochemical Properties of Inclusions and Virions

Most inclusions extracted from insect tissues and purified by centrifugation through sucrose gradients reached equilibrium in 55 to 60% sucrose. When collected and examined under the light microscope using phase contrast optics, these inclusions appeared refractive to light, oval to ellipsoid, and up to 14.4  $\mu\text{m}$  long by 11.9  $\mu\text{m}$  in diameter (Figure 8). Some inclusions reached equilibrium at lower density levels in the gradients, indicating differences in the densities. These latter inclusions were considered as being incomplete or immature.

When inclusions were treated with various hypotonic sodium solutions, they expanded to about 3 X normal size, lost the light refractive characteristic, and possessed homogeneous periphery around an internal particulate area (Figure 9). The particles, virions, appeared more resistant to degradation than the matrix of the inclusions in sodium solutions. Electron microscopy of thin sections of mature inclusions confirmed that the particles were virions and that, under electron microscopy, the mature inclusions were up to 11.5  $\mu\text{m}$  long by 9.8  $\mu\text{m}$  wide.

The occluded virions of APV appeared brick-shaped and were from

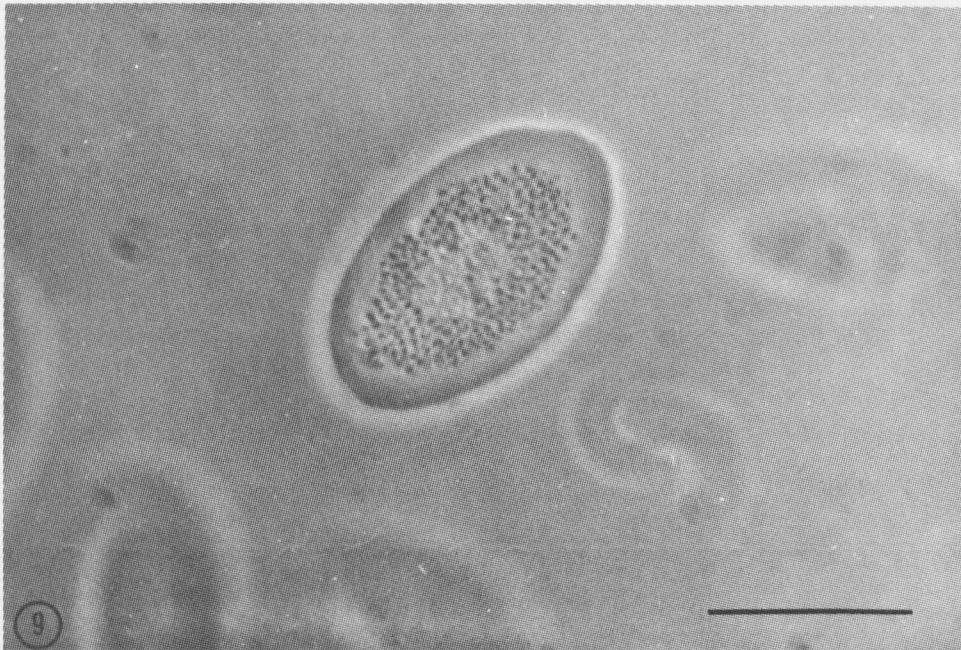


Figure 9. An expanded inclusion of APV after treatment for 5 minutes with 10% sodium dodecyl sulfate (SDS).  
Line Scale = 21  $\mu$

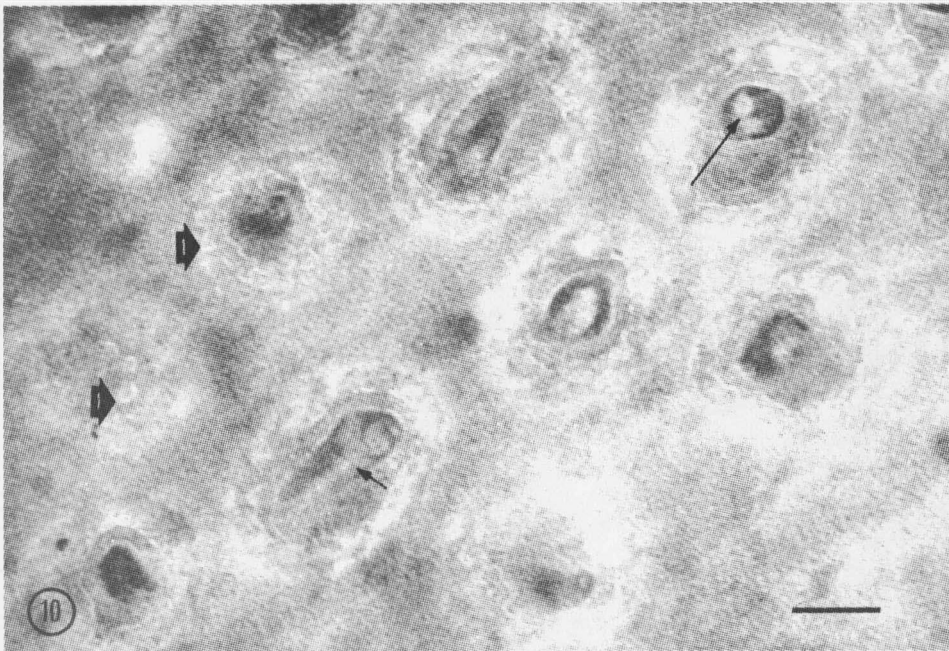


Figure 10. Electron micrograph of virions within an inclusion. The electron dense cores of the virions (thin arrows) contain less dense, rod-like, DNA inner cores and are surrounded by a trilaminar membrane with a capsid consisting of spherical subunits (heavy arrows) and an outer "halo".  
Line Scale = 120 nm

273 to 336 nm long by 168 to 209 nm wide (Figure 10). These measurements included the halo which is an outer less-electron-dense area of the virion. The core portion of the virion, consisting of the nucleic acid surrounded by a protein coat, measured 189 nm long by 47 nm wide. The central nucleic acid core, which contained the large nucleic acid strands that appeared less electron dense (Figure 10), measured 168 nm long by 34 nm wide. Based on examination of the configuration of the nucleic acid strand in both longitudinal and cross sections of virions, it appears as a long cylindric structure that is folded 3 or 4 times. The protein coat consisted of an outer lighter halo having a wavy appearance, with a basal bilayered structure that appeared similar to the unit membrane of cells. The protein coat of the virus was approximately 13.48 nm thick. Freed virions were more ovoid when examined after being extracted from inclusions by a sodium digest and then shadow casted with platinum for electron microscopic examination (Figures 11, 12, 13). By measuring the length of the shadow produced by a 3:1 angle ratio, it was determined that these virions were about 136 to 249 nm in diameter. Virions that were recovered from preparations of inclusions following sodium treatment reached equilibrium in 50 to 55% sucrose. In contrast, virions recovered from homogenates of tissues at 14 days post-inoculation, thus nonoccluded virions, were less dense and reached equilibrium at

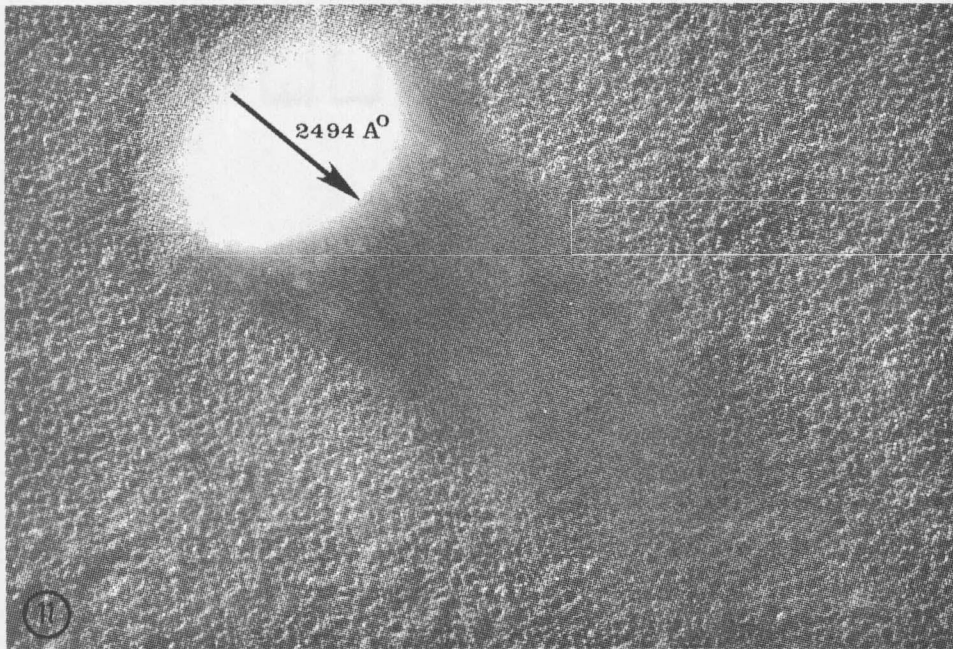


Figure 11. Electron micrograph of a shadowed virion of APV released from inclusions by sodium digest. Arrow shows direction of shadowing and diameter of virions as determined from the shadow.

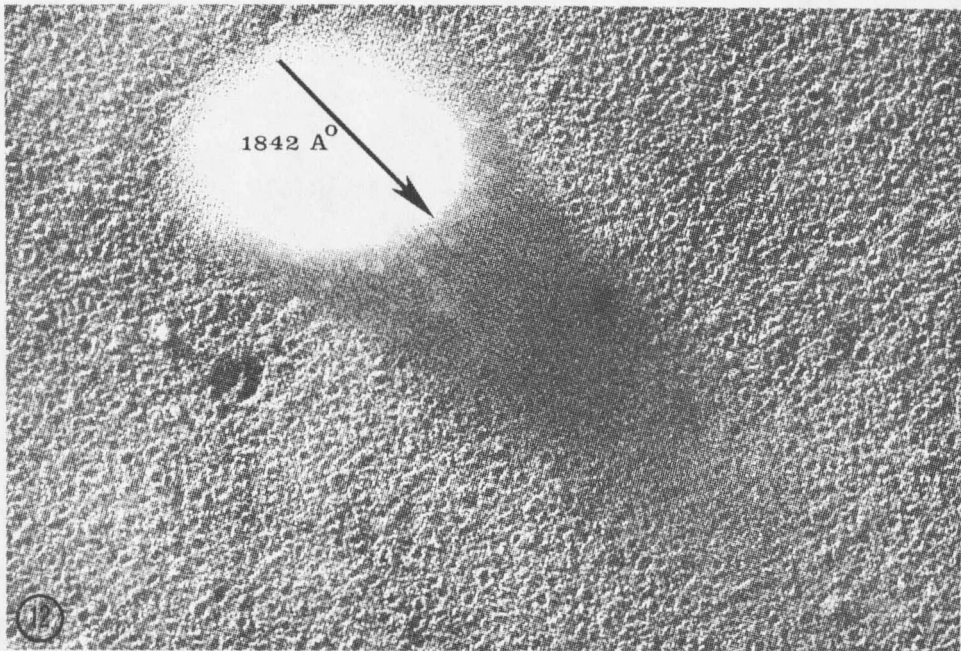


Figure 12. Electron micrograph of a shadowed virion of APV released from inclusions by sodium digest. Arrow shows direction of shadowing and the diameter of the virion as determined from the shadow.

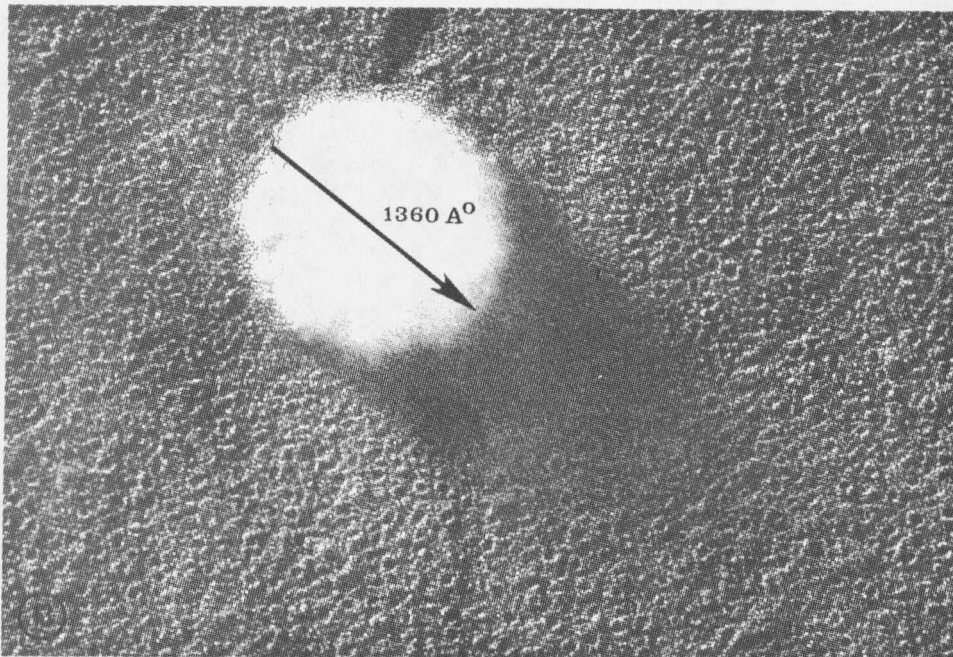


Figure 13. Electron micrograph of a shadowed virion of APV released from inclusions by sodium digest. Arrow shows direction of shadowing and the diameter of an uncharacteristic virion as determined from the shadow.

an interface of 30 to 35% sucrose.

In preparation of thin sections of inclusions for electron microscopy, the nucleic acid core stained readily with uranyl acetate and appeared electron dense indicating the presence of deoxyribonucleic acid (DNA). This was confirmed by staining using the Feulgen technique. Simultaneous staining of an RNA virus, which did not accept the stain, was used as a check procedure. Furthermore, treatment of the virions with fluorochrome acridine orange also resulted in a positive reaction which established that the DNA within APV virions is double-stranded.

The technique reported by Gafford and Randall (5) was effective for the extraction of the DNA from APV virions. Although analysis of the molecular weight was not completed, the observation that APV-DNA moved through polyacrylamide gels more slowly than the DNA from the lambda bacteriophage, (M.W.  $3 \times 10^7$  daltons), which was electrophoresed simultaneously, indicated that the molecular weight of APV-DNA is very large.

Based on the degradation of inclusions by sodium dodecyl sulfate (SDS), sodium hydroxide, sodium carbonate, and other protein degrading agents, and formation of precipitate when these preparations were treated with 10% trichloroacetic acid (TCA), protein appeared to be the principal constituent of inclusions. The amount of protein in

inclusions and virions was determined colorimetrically with the Folin-Ciocalteu protein reagent. Figure 14 shows the standard curve derived from bovine serum albumin which was used for indirect calculations of the amount of protein in inclusions and virions shown in Table 1. Accordingly, these estimates established that about 87 percent of all protein is inclusion protein and the remainder is protein associated with the virion. The presence of lipids in inclusions also was established when infectivity was altered by chloroform-ether pretreatment and the loss of some cross activity in antigen-antibody reactions when the inclusion preparations were extracted with chloroform. Although the amount of lipid was not determined, it is expected that most of it is associated with the intimate membranes of the virions and represents a very minor part of the inclusions.

#### Electrophoretic Patterns of Proteins of the Entomopoxviruses from Grasshoppers

Although the electrophoretic patterns of the proteins from Arphia poxviruses inclusions (APV-I), grasshopper inclusion body virus inclusions (GIBV-I), and Phoetaliotes poxvirus inclusions (PPV-I) were quite similar, some definite differences were observed (Figure 15). One protein band (6a) was seen in APV profiles that was not seen with GIBV or PPV and another protein band (2a) was seen only in PPV profiles. Band 5 in PPV profiles exhibited greater electrophoretic mobility than

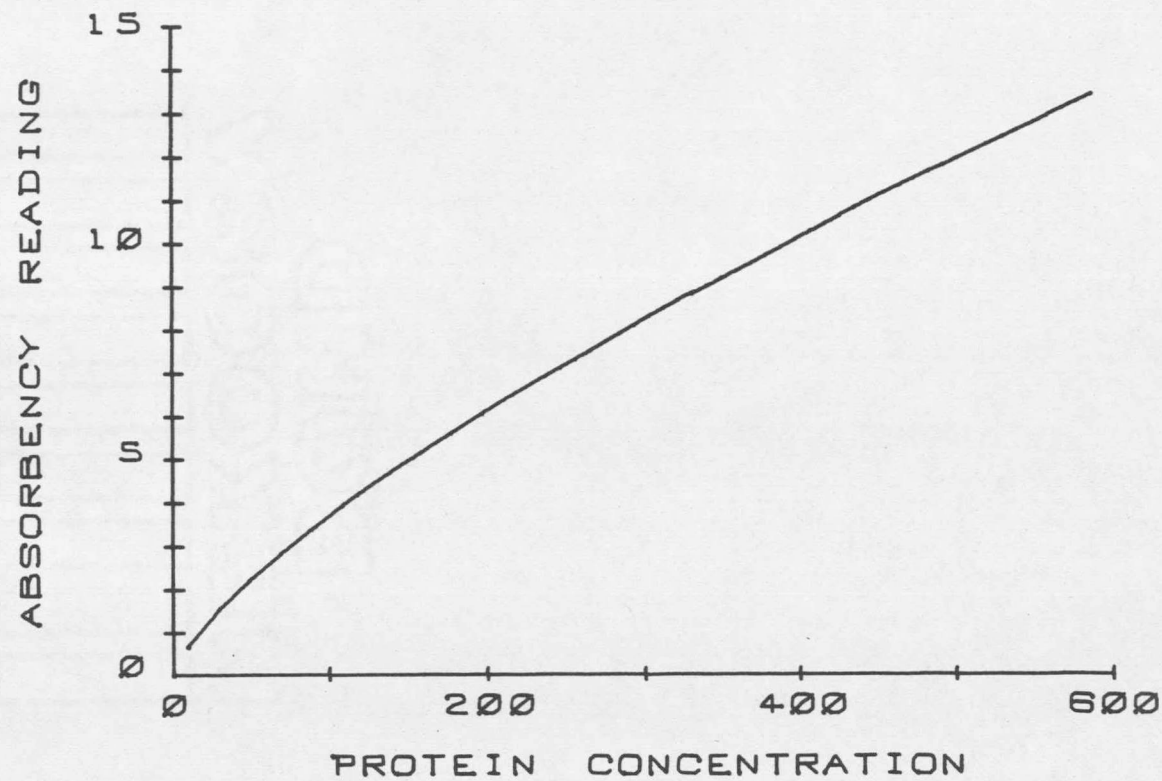


Figure 14. Graph of protein concentrations of bovine serum albumen used as a standard for determining the protein concentrations of inclusions and virions of APV.

Table 1. Protein concentration of the inclusions and purified virions of the Arphia poxvirus (APV) as determined by the modified Lowry technique (14, 21).

| No. Inclusions/<br>ml                                | Average<br>Absorbance (Y) | Protein Conc.<br>$\mu$ l/ml |
|------------------------------------------------------|---------------------------|-----------------------------|
| Inclusions                                           |                           |                             |
| A. $6.56 \times 10^6$                                | 1.497                     | 669.4                       |
| B. $3.05 \times 10^6$                                | 1.022                     | 397.1                       |
| C. $1.53 \times 10^6$                                | .700                      | 236.6                       |
| D. $7.55 \times 10^5$                                | .544                      | 167.6                       |
| Virions Purified from Inclusion Preparations A. & B. |                           |                             |
| A.                                                   | .376                      | 101.1                       |
| B.                                                   | .326                      | 83.0                        |

in profiles of either APV or GIBV. Less discreet differences observed in the mobility of proteins from these viruses were considered as nonsignificant because of the possible effects of slight differences in the gels from one run to the next. When inclusions were treated with sodium solutions and the virions were removed by centrifugation, band 1 and 2 no longer formed in the protein profile of the inclusions. Thus, these proteins undoubtedly were derived from the viral particles and possibly represent coat and core proteins. Also some differences were observed in proteins of the inclusions with virions when electrophoresis occurred following pretreatment of the samples in 10% SDS overnight (Figure 15C). Although the number of bands in each of the profiles differed, the most significant difference appeared to be in the separation of the upper bands which probably represented the proteins of the virions.

#### Antigenic Differences Among Entomopoxviruses from Grasshoppers

Studies of the antigenic properties of APV, GIBV, and PPV by gel diffusion techniques (Ouchterlony) showed some very distinct differences between viruses. Definite reactions of non-identity were observed when either GIBV-I or APV-I were run simultaneously against both anti-inclusions (Figure 16). The most apparent of these reactions resulted in the formation of an outer band labelled A. This indicated that APV serum contained antibodies to a protein present in relatively

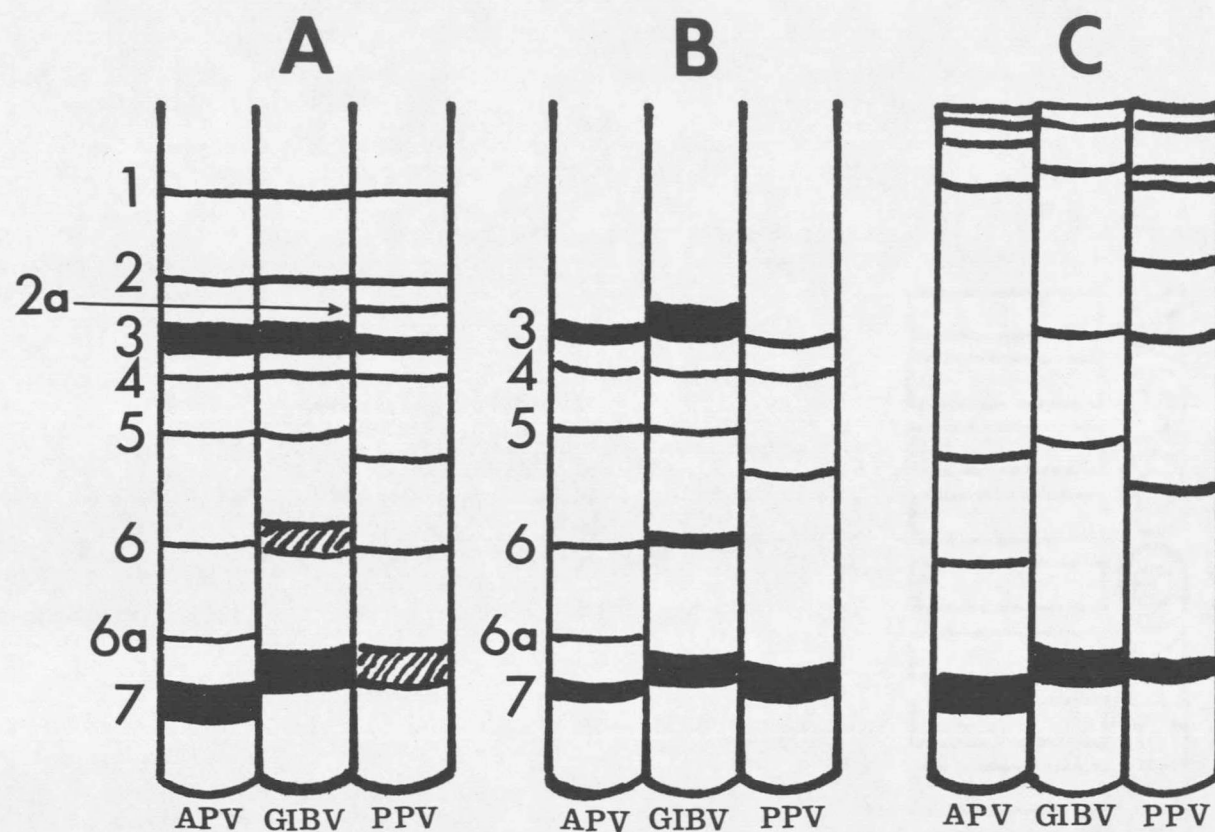


Figure 15. Diagram of the polyacrylamide gel electrophoresis profiles of proteins from inclusions of APV, GIBV, and PPV. A. Profiles of inclusions with virions digested with 0.1% sodium carbonate, 0.1% sodium thioglycolate. B. Profiles of inclusions without virions digested with 0.1% sodium carbonate, 0.1% sodium thioglycolate. C. Profiles of inclusions with virions digested with 10% SDS.

large concentrations in APV-I but which was present only in minimal amounts in GIBV-I. Reactions of non-identity also were suggested in the comparison of inner bands B with B' and D with D'. Band C appeared similar in these cross reactions and probably represented the presence of an identical or very similar protein determinant in the inclusion.

When anti-APV-I and anti-GIBV-I sera were absorbed against normal fat in order to remove host determinants most of the bands disappeared (Figure 17). The remaining band or bands appeared to be D with D' which again formed in a manner showing a reaction of non-identity with inhibition between APV and GIBV sera. When either APV-I or GIBV-I that were extracted with chloroform were run against the anti-inclusion sera of either virus, the outer band A was lost, indicating that it was formed by a lipo-protein determinant.

Serum was prepared against the virions of APV (APV-V) and was tested for reactions to both purified APV-V and GIBV-V. When APV-V were reacted to this serum two bands resulted, both being rather sharp (Figure 18), but when GIBV-V were reacted to this virion antiserum, only one band that was not nearly so distinct occurred along with an inner diffuse band. Reacting both purified APV-V and GIBV-V simultaneously to this virion serum resulted in the formation of an inner band of identity associated with both virions, a wide outer band associated with GIBV-V only, and three distinct bands associated only

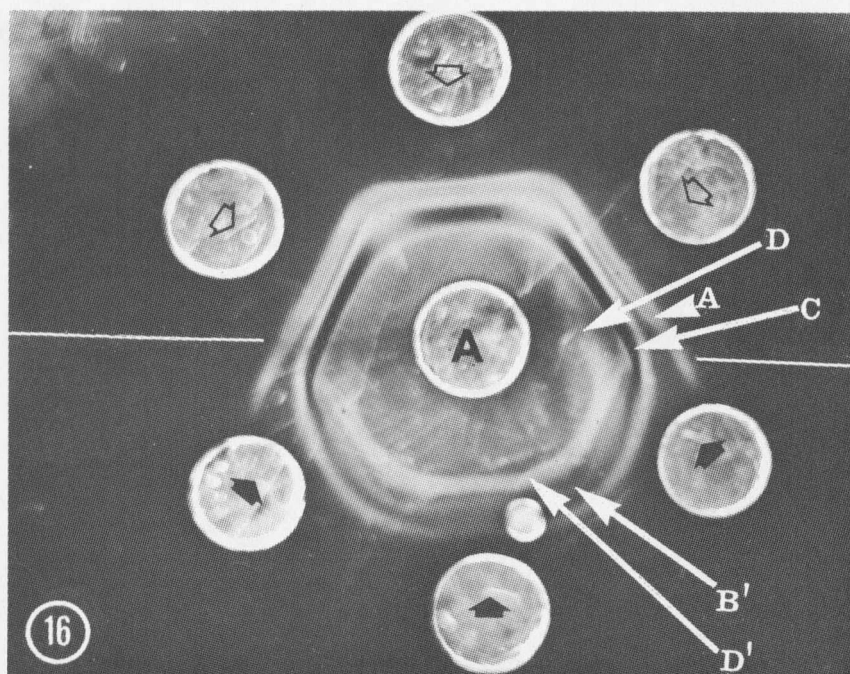


Figure 16. Double-diffusion patterns of GIBV inclusions (A) versus both APV (hallow arrows) anti-inclusion serum and GIBV (solid arrows) anti-inclusion serum.

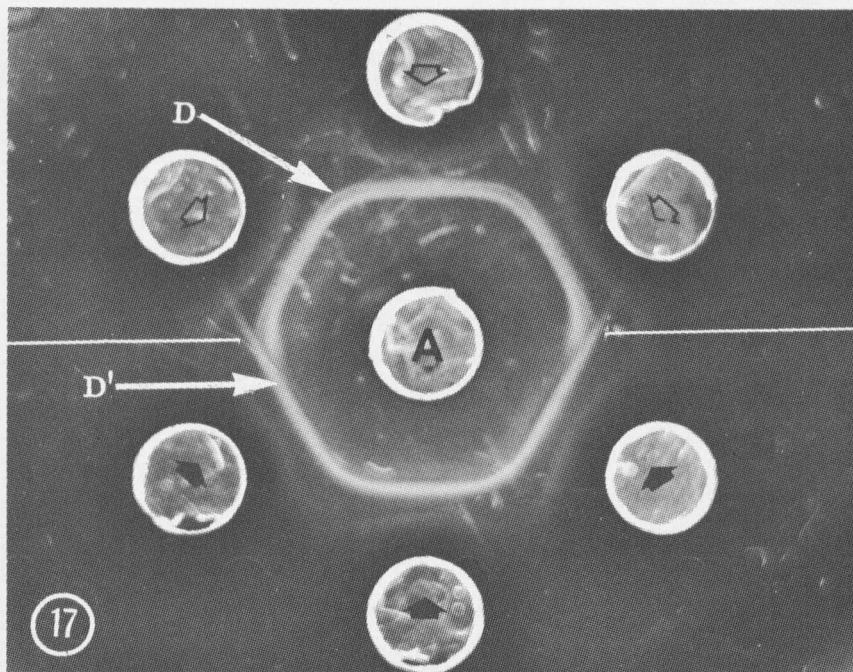


Figure 17. Double-diffusion patterns of GIBV inclusions (A) versus both APV anti-inclusion serum (hallow arrows) and GIBV anti-inclusion serum (solid arrows) after absorption of the sera with normal grasshopper fat tissue.

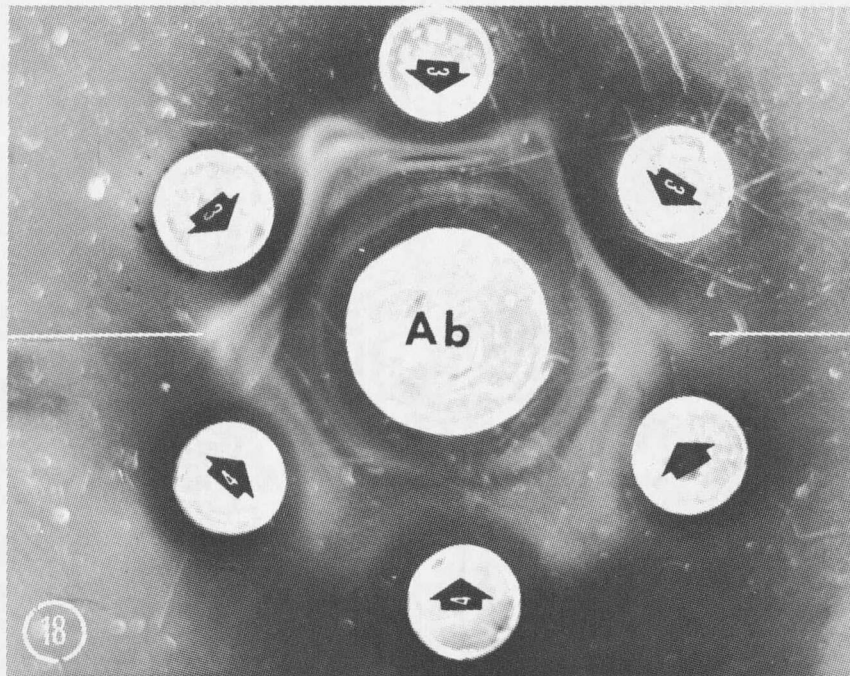


Figure 18. Double-diffusion antigenic patterns of virions from both APV (Arrows 3) inclusions and GIBV inclusions (arrow 4) versus antiserum to APV virions.

with APV-V. These reactions suggest that APV and GIBV possess one identical or very similar protein determinant, but that all other determinants are different.

## Chapter 4

### DISCUSSION

Entomopoxviruses have been characterized as large inclusion body viruses, the virions of which are brick-shaped and measure about 350 nm long by 250 nm wide. They are DNA viruses that replicate by budding from virogenic stroma after which they undergo differentiation before and after being occluded within the proteinaceous inclusion that measures from 10  $\mu$ m to 24  $\mu$ m in diameter (2, 9, 19). The virions of Arphia poxvirus (APV), an inclusion body virus isolated from Arphia conspersa, were shown to be about 337 nm long by about 209 nm wide and to contain double-stranded DNA. Also, the virions of APV possessed a tri-laminar intimal coat surrounded by a middle layer of spherical bodies and by an outer halo coat such as has been described for some entomopoxviruses (9, 19). The inclusion bodies were composed mainly of protein and measured up to 11.9  $\mu$ m in diameter. The replicative process appeared similar to that reported in other entomopoxviruses (6, 26). Based on these criteria, APV has been assigned to the entomopoxviruses.

APV is one of three entomopoxvirus isolated from grasshoppers. The grasshopper inclusion body virus (GIBV) was isolated from Melanoplus sanguinipes and was characterized as an entomopoxvirus (11, 12). More recently a third probable entomopoxvirus, the

Phoetaliotes poxvirus (PPV) was isolated from the grasshopper Phoetaliotes nebrascensis. Although these viruses share many similar characteristics, such as size and structure of inclusions and virions, as well as replication in the cytoplasm of fat cells, other gross pathological, chemical, and antigenic characteristics established that they are distinctly different from one another. For example, the host range of APV was limited to species of the subfamily Oedipodinae whereas species of the subfamily Cyrtacanthacridinae, that were tested, were refractory to infection. In contrast, GIBV and PPV appear infectious to species of the subfamily Cyrtacanthacridinae with some cross infectivity between the two within this subfamily, but with neither being infective for species in the subfamily Oedipodinae. Differences between the viruses were apparent when inclusions of the three viruses were subjected to protein electrophoresis. The proteins from purified virions of the three viruses showed equal electrophoretic mobility, but the inclusion proteins seem to be slightly different. Serological cross reactions seem to confirm that the two proteins associated with the virions of APV, which may be core and coat proteins, have some similarities to the proteins in GIBV and PPV. However some differences appeared (after treatment in 10% SDS), which suggested possible genetic differences. More discreet differences were evident in the patterns of the inclusion

that showed that the inclusions of the three viruses differed in their constituent proteins. Although these differences in proteins suggest differences in the viruses, it should be pointed out that such an interpretation is valid only if the makeup of the inclusions is viral directed.

The serological comparisons of protein determinants in the inclusions showed that APV and GIBV possessed some determinants that were virus directed. This was evident from the reaction of non-identity with inhibition between inclusions and homologous antisera after absorption with normal host tissue to remove the host determinants. These analyses did not establish that the protein determinants that caused this reaction were the same as those that exhibited different mobilities in the electrophoretic patterns. Nevertheless, these reactions, together with cross reactions between the virions of APV and GIBV with antiserum prepared against APV virions that showed reactions of non-identity between APV antiserum and GIBV virions, establish that discreet differences exist between these viruses.

Although the general pattern of the replication of APV appears similar to the patterns that have been reported for other entomopoxviruses (6, 26), certain points brought out in these studies bear mentioning. For example, virogenic stroma reported by Henry et al. (12) in the replication of GIBV appeared larger than those observed

in the replication of APV. The consideration that this may be a host induced difference, since the hosts of APV differ from those of GIBV, appears less likely following observations that the sizes of the APV stroma appear similar in both Arphia conspersa and Camnula pellucida. Thus, this size difference may represent an additional difference between APV and GIBV. Goodwin and Filshie (6) reported that the granular material within the stroma stained differently with uranyl acetate and that both light and dark staining was evident within a single stroma. This also was observed in studies of APV and consideration was given to the presence of both protein and DNA pools within the stroma. It is apparent from the nature of the triple-layered membrane that lipid determinants may be present in virions. This lipid may be derived from the host and protein of the membrane may be viral directed.

A number of studies relating to the replication process of entomopoxviruses, as well as observations of structural similarities, have demonstrated the close relationships between the entomopoxviruses of insects and the vertebrate poxviruses. This close relationship was reiterated by Stoltz and Summers (26) with their suggestion that the basic shell structure around the virion was common to all poxviruses, including entomopoxviruses, and that it is a single unit membrane surrounded by hexagonal subunits randomly distributed on the surface.

Based on the surface structure of APV virions, examined as ultra-thin sections or as shadow cast preparations under electron microscopy, these subunits appear spherical and are not evident on the nonoccluded virions present in the cytoplasm of infected cells. Rather these spherical subunits appear upon occlusion of the virion. Therefore, this possibly represents a difference between the vertebrate poxviruses and entomopoxviruses in the origin and development of the viral capsid. Goodwin and Filshie (6) postulated the occurrence of a "maturation" process in virions being occluded. Differences in bouyant density between freed and occluded APV virions might be the result of the deposition of the spherical subunit membrane and the halo protein onto the triple layered membrane. These steps, along with a further compaction of the virions on occlusion, could account for the maturation process.

The development of the inclusion body by the entomopoxviruses represents the most obvious and distinct difference between entomopoxviruses and vertebrate poxviruses. This then presents the question as to whether the inclusion is host directed or virus directed. Based on these studies with APV, it appears that both the host and the virus contribute to the development of the inclusion. In fact, these studies indicated that at least one protein in the inclusion was the same as one of two proteins associated with the virions. Whether or

not this protein is a structural constituent of the inclusion or merely a contaminant acquired during development could not be ascertained. Nevertheless, these and other aspects evident in this study of APV show that structure, development, and host cell interactions of entomopoxviruses are very complex and that very specialized and detailed studies will be required to provide an understanding of entomopoxviruses.

## Chapter 5

### SUMMARY

The Arphia poxvirus (APV) was isolated from the grasshopper Arphia conspersa and was transferred to the grasshoppers Camnula pellucida and Xanthippus corallipes. Gross symptomatology associated with infections included abdominal swelling and discoloration, ataxia, slowed growth, and death. Infections were restricted to fat bodies which showed increased hypertrophy during pathogenesis. Replication of the virus occurred in the cytoplasm with immature virions budding from granular amorphous stroma after which they underwent internal structural differentiation followed by occlusion in protein matrix inclusion bodies. The inclusions measured up to 14.4  $\mu\text{m}$ , were refractive to light, and expanded when treated with various protein degrading chemicals. Occluded virions measured about 300 nm in length and consisted of a nucleocapsid core surrounded by a tri-laminar membrane with a capsid composed of spherical protein subunits.

Comparisons of the electrophoretic patterns of the proteins of APV with the proteins of the grasshopper inclusion body virus (GIBV) and Phoetaliotes poxvirus (PPV), both of which also exhibit characteristics of entomopoxviruses, established that these viruses contained many proteins with the same or very similar electrophoretic mobilities, but also some proteins with different mobilities. Also, serological

cross reactions between APV and GIBV established the presence of protein determinants that were similar, or identical, and some that were different. These results, along with differences in the infectivity for different species of grasshoppers provided the basis for concluding that APV was distinct from GIBV or PPV.

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