# Temporal Events in the Invasion of the Codling Moth, *Cydia pomonella,* by a Granulosis Virus: An Electron Microscope Study

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The replication cycle of the granulosis virus of *Cydia pomonella,* the codling moth, was studied at the cellular and tissue level. Membranelike complexes were observed forming within the remnants of the nucleolus in the cytoplasm of infected cells. Differences in cell polarity relative to the sites of virus entry assembly and budding as well as differences in the temporal aspects of replication were observed between midgut, fat body, and epidermal cells. The progressive spread of virus throughout larval tissues was studied at 24, 32, 48, 56, and 72 hr postinfection. The basal lamina seemed to be an effective barrier for the release of budded progeny virus into the hemocoel and large numbers of budded virus were produced. © 1987 Academic Press, Inc.

KEY WORDS: Codling moth; *Cydia pomonella;* granulosis virus; baculovirus replication.

## INTRODUCTION

The codling moth, *Cydia pomonella,* is susceptible to a baculovirus of subgroup B of granulosis viruses. This granulosis virus is polytrophic, infecting many of the tissues of the codling moth, and is highly pathogenic. Because the codling moth is an economically important pest, widespread interest in the use of this virus as a biological insecticide has developed. Not only have there been extensive field application tests (Falcon et al., 1968; Hüber and Dickler, 1975; Jaques et al., 1977) but considerable work has been done on the physical characterization of the codling moth granulosis virus (Croizier and Meynadier, 1973; Roberto, 1980; Harvey and Volkman, 1983).

The codling moth granulosis virus (CMGV) was first described by Tanada (1964). Subsequently, electron microscope studies have been made of individual aspects of the replication process (Stairs et al., 1966; Tanada and Leutenegger, 1968; Falcon and Hess, 1985). None of these studies have thoroughly addressed the cytopathology of CMGV in the entire insect at the cellular and tissue levels although several light microscope studies have partially dealt with this issue (Wäger and Benz, 1971; Benz and Wäger, 1971). In this study we have attempted to obtain an overall view of the pathology of CMGV as it spreads through its host both at the cellular level and within different tissues by examining differences in susceptibility, viral replication, and pathogenicity.

## MATERIALS AND METHODS

Fifth-instar larvae of *C. pomonella* were fed one of three granulosis virus inocula. These were supplied by J. P. Harvey and had been previously biochemically characterized and designated as CPGV-MB, CPGV-MD, and CPGV-R (Harvey and Volkman, 1983). After initial observations, it was determined that all specimens produced similar pathogenicity at the electron microscope level and therefore all specimens were viewed collectively within each time interval studied.

Larvae were fed 2-mm cubes of diet on which 20 μl of CMGV at a protein concentration of 24.5 to 25 μg/ml had been deposited. Control larvae were fed diet inoculated with 20 μl of distilled water. Additional control larvae were fed diet alone.

After the diet cube was completely ingested (between 15 min and 1 hr), fresh diet was added and timing of the experiment for that larvae began. Twelve to fifteen larvae were maintained at 20°C and sampled at 24, 32, 48, 56, and 72 hr postinoculation in a series of two experiments. Tissue, including the midgut, fat body, and epidermis, were immersed in 2.5% phosphate-buffered glutaraldehyde, $pH$ 7.0, for 1 to 2 hr and subsequently postfixed in 1% osmium tetroxide (phosphate buffered, $pH$ 7.0) for 1 hr. Samples were embedded in Araldite 6005 after dehydration in ethanol. Sections, stained with saturated aqueous uranyl acetate followed by lead citrate, were examined in a Philips EM 300 electron microscope.

## RESULTS

*Controls*

The tissues of the codling moth as well as other lepidopterous larvae are in a dynamic state of flux, continuously reflected by changes in their respective morphology. The cells in any tissue are a mixed population of functional types and these cells in turn are of diverse ages reflected in different physiological states. What is described here is a generalized view of the control tissues.

In the codling moth, the midgut cells were observed to be of four general types. The most frequent was the typical columnar cell the inclusions of which varied in structure due to the position within the gut and probable function. In general, the nuclei of the cells were located in the central to basal two-thirds of the cell. These nuclei varied in electron density, as did the nuclei throughout the insect (Fig. 1). Apically the cells were bounded by microvilli and basally they were highly infolded. These infoldings extended upward into the basal one-third or so of the cell and ended in close proximity to the nucleus. The cell rested on a basal lamina to which it was attached by periodic hemidesmosomes. The

cells contained varying amounts of smooth and rough endoplasmic reticulum. Mitochondria with the tubular-type of cristae predominated. Also present were the Golgi apparatus, primary and secondary lysosomes, and various electron-dense bodies. The apical area of the cell just below the microvilli was generally clear of organelles and contained a fibrous zone of filaments extending from the microvilli. Within this zone, coated pits and pinocytotic vesicles were observed at the base of the microvilli. Interspersed among the columnar cells were goblet cells. In these cells the apical microvilli frequently were seen to abut and junctions were formed between the villi. The majority of goblet cell microvilli were lining a chamber invaginated into the apical portion of the cell. The microvilli lining the chamber each contained a mitochondrion with tubular cristae. Basal infoldings were maximized in this cell type. In addition, lateral invaginations of the plasma membrane occurred, creating a labyrinth of cytoplasmic extensions in the basal two-thirds of the cell. Between the columnar and goblet cells were nidi of regenerative cells. These rested on the basal lamina and did not extend to the luminal side of the gut. These cells were small and the cytoplasm was usually quite electron dense as were the nuclei. The cytoplasm was generally undifferentiated in that it contained only a few mitochondria and some rough endoplasmic reticula. Also present in the midgut were occasional cells that were apparently endocrine in nature. These cells were thin, contained many electron-dense granules with an irregular outline, did not appear to have basal infoldings, and were not observed to open to the lumen of the gut. Tracheoles were observed within the basal portions of the midgut as were neural axons and cells containing neurosecretory granules. Beneath the basal lamina the midgut was invested with a layer of tissue containing muscles, trachea, and nerve cells.

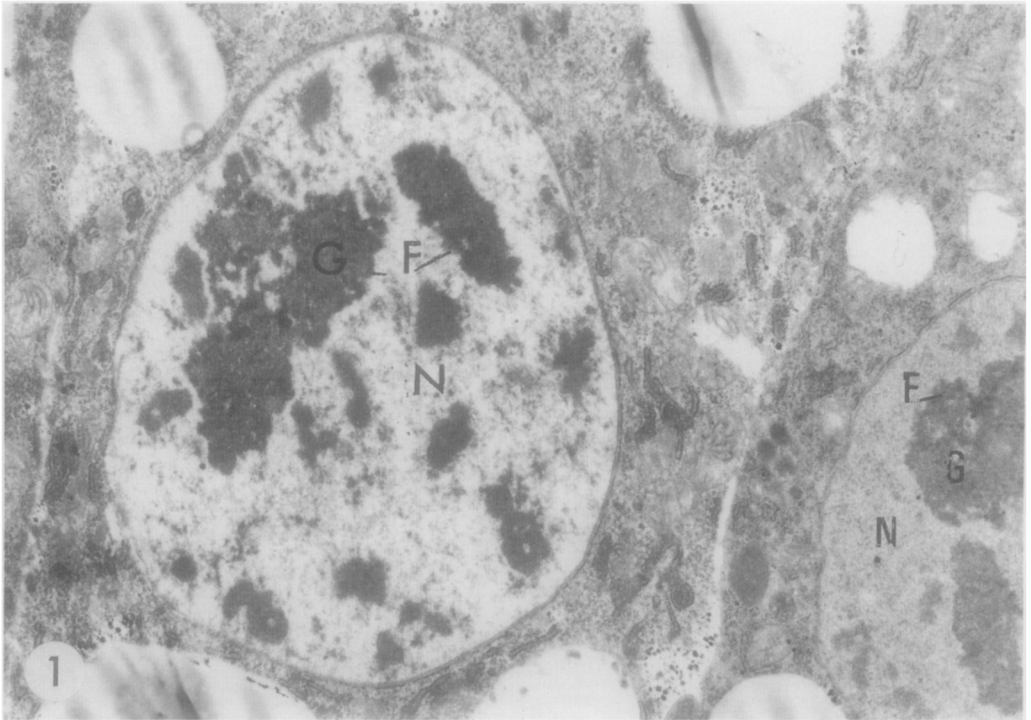The midgut cells were interconnected by

FIG. 1. Nuclei (N) of the fat body are shown which differ in morphology. F, fibrillar component of nucleolus; G, granular component of nucleolus. ×13,700.

FIG. 2. In this midgut nucleas early in infection the nuclear membrane has begun to disassociate (arrows). The nucleolus has enlarged and the granular (G) and fibrillar (F) components are evident. Nucleocapsids (V) are present. ×11,340.

smooth septate junctions interspersed with desmosomes. These junctions appeared to link the cells in their apical one-third areas. Some gap junctions were also observed. The mid and basal regions of the gut were open because of lack of intercellular junctions and the extensive basal and lateral infoldings of the cells. Basally they were held in place by patches of hemidesmosomes which attached to the basal lamina.

The fat body of control larvae appeared fairly uniform in structure in this instar. Both sheets of fat body, that associated with the epidermis and that free in the hemocoel near the gut, were examined. In some cases the fat cells were one cell thick with a basal lamina apparent on either side. In other places, the fat body appeared to be thicker. The cells contained multiple lipid droplets of various sizes, multivesicular bodies, some vacuoles, small protein granules, and scattered deposits of glycogen. Mitochondria contained thin cristae in contrast to the tubular cristae observed in midgut cells. The rough endoplasmic reticulum was distributed in moderate amounts throughout the cytoplasm. Nuclei in fat body cells were of varying densities, probably reflecting differences in physiological state or age (Fig. 1). Cells were interconnected in spots by desmosomes and gap junctions. Within a sheet of fat body tissue, some large spaces were observed occasionally between adjacent cells. These spaces were apparently open to the hemolymph since the cell surfaces were covered with a basal lamina; however, the apical and basal portions of the cells were interconnected in the plane of sectioning. The surfaces of the fat body open to the hemocoel were covered by a basal lamina which was attached to the cells by hemidesmosomes. The basal lamina was continuous with the basal lamina of closely associated cells such as tracheal and neural cells. In the fat body associated with the epidermis, the confluence of their individual basal lamina resulted in especially thick or multiple investing layers.

The epidermis (hypodermis) is a single layer of cells immediately beneath the cuticle responsible both for secretion of new cuticle and digestion of old cuticle. The apical cellular surface was differentiated into short microvilli at the tip of each was a plaque which bound it to the cuticle. Beneath the microvilli was a small clear zone in which some vesicles were occasionally observed. These vesicles contained a flocculent material. The nuclei of the epidermal cells were generally round to oval and occurred in the more basal region of the cell. Scattered throughout cells were electron-dense granules with a difficult to resolve substructure similar to multivesicular bodies. Also present in the cytoplasm in variable numbers were vacuoles which contained a flocculent material similar to that observed in the vesicles associated with microvilli. The density of the epidermal cells was variable and apparently related to the amount of rough endoplasmic reticulum present in the cell. Many cells were observed to have large deposits of glycogen in the basal region of the cell. At times these deposits had electron-dense granules or electron-dense lamellar bodies associated with them. Mitochondria contained tubular cristae and in some cells the mitochondria were quite electron dense. The basal portions of the epidermal cells were relatively flat, resting on the basal lamina with periodic hemidesmosome attachments. The lateral edges of the adjacent cells were interdigated with varying degrees of complexity. Epidermal cells were held tightly together along their entire lateral surface with pleated septate junctions along with desmosomes and gap junctions.
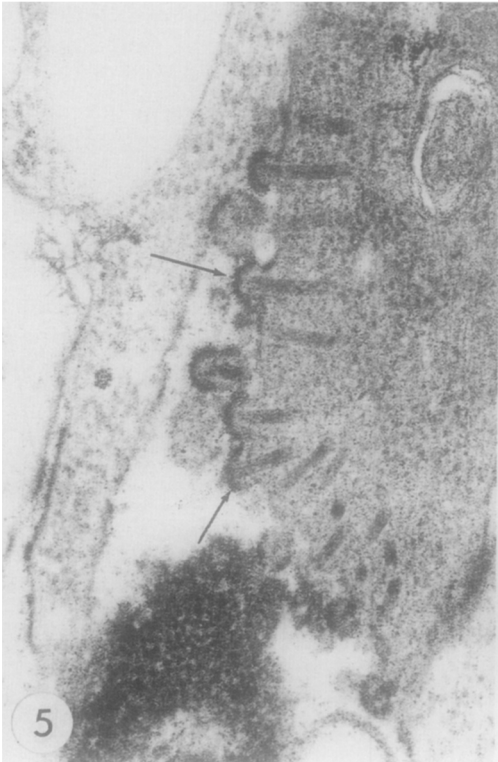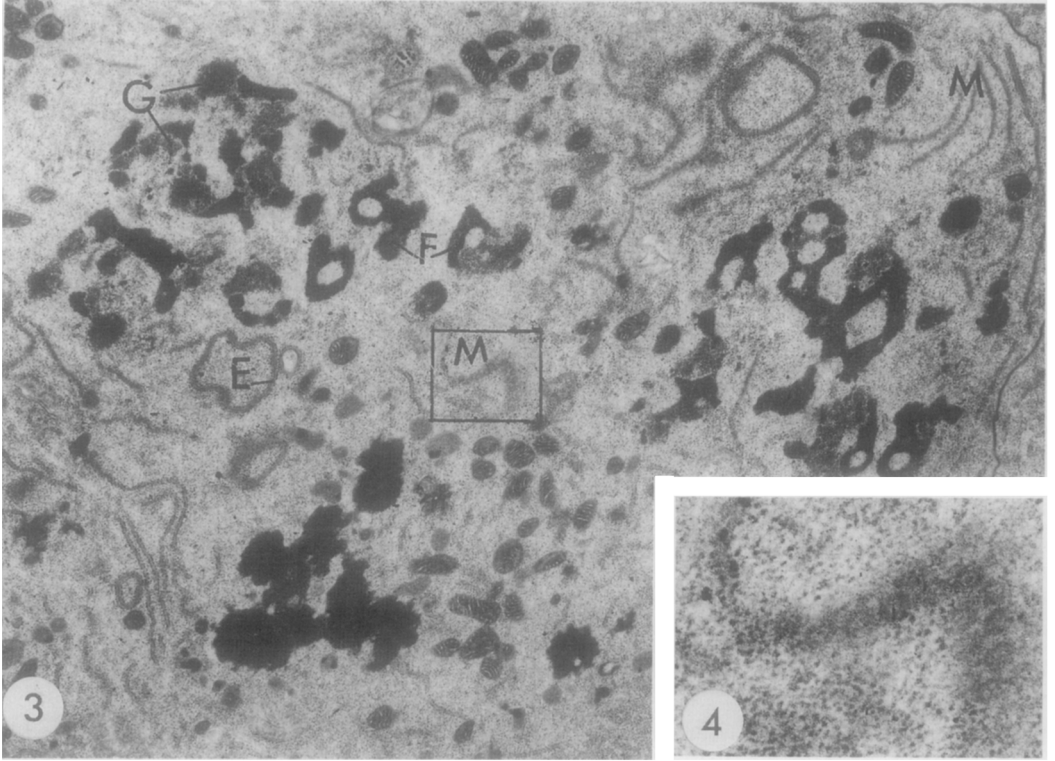
## Cellular Replication of CMGV

The precise sequence of granulosis virus development in vivo is difficult to determine because of unknowns such as the exact time infection occurred, etc. However, a general overview can be obtained by studying many cells in various stages of

infection. In the codling moth, the first overt signs of infections appeared to be nuclear "clearing." In this case the chromatin marginated as well as the nucleolar complex (Fig. 2). The granular component of the nucleolus appeared to be very prominent in the cleared nucleus, suggesting an increase in overall size. Associated with the main nucleolus initially and then later dispersed throughout the nucleus were numerous electron-dense fibrous bodies, the fibrillar component of the nucleolus (Fig. 2). The nuclear pores appeared to increase in numbers and became very apparent along the entire nuclear envelope. In the central areas of the nucleoplasm, clear tubules approximately 40 nm in diameter appeared. These tubules were presumably empty capsids. Subsequently an increase in electron density within the capsids occurred and nucleocapsids became apparent throughout the central regions of the nucleus (Fig. 2). The number of nucleocapids observed within a nucleus at the time the nuclear envelope fragmented was variable. The nuclear envelope at this point was composed predominately of interconnected nuclear pores and in some areas was almost devoid of ribosomes. Areas of the nuclear envelope appeared to be closely associated with groups of cytoplasmic microtubules. The nuclear envelope fragmented into multiple pieces which stayed near the nuclear area of the cell and appeared similar to sections of annulate lamellae (Fig. 3). Tangential sections revealed these as sheets of nuclear pore complexes (Fig. 4). Those nucleocapsids already formed in the nucleus and released at the time of nuclear envelope fragmentation appeared to be the first nucleocapsids to reach the plasma membrane for subsequent budding. No other method of nucleocapsid release into the cytoplasm was observed in this study. No nucleocapsids were found in the cytoplasm unless a break in the nuclear envelope was present. Nucleocapsids were seen with the conical or hemispherical end perpendicular to the plasma membrane. The blunt end of

the nucleocapsid was never observed in this association. The plasma membrane was thickened in the area of contact (Fig. 5). In certain instances, fibers were observed connecting the conical end of the virus capsid with the thickened membrane. Budding proceeded with the nucleocapsid becoming surrounded by the altered plasma membrane and terminated with the pinching-off of an enveloped virion into the space between the plasma membrane and the basal lamina or into the intercellular space (Fig. 6). Some of the virions had spikelike projections, the peplomers, at one end. It was not possible to ascertain whether all budded viruses had these peplomers. The peplomers occurred as a bulb on the same end of the virion as the conical portion of the capsid.

While budding was proceeding, further proliferation of nucleocapsids continued in the portion of the cell which the nucleus once occupied. This virogenic area remained the same throughout viral replication. Nucleocapsids continued to move to the periphery of the cells. Some of these nucleocapsids budded, accumulating beneath the basal lamina and in the intercellular space. Other nucleocapsids appeared to congregate within envelope formation areas. These areas were characterized by the presence of masses of vesiculated membranous profiles. The conical or hemispherical end of the nucleocapsid again aligned perpendicularly to a membranous profile. Nucleocapsids were seen with variable degrees of encompassing membrane as well as completely enveloped virions. Capsule formation began after the first enveloped nucleocapsids appeared. All stages of viral replication were visible in the cell at this point. Capsule formation was first observed as a small deposit of proteinaceous material (granulin) on one area on the virus envelope. Nucleocapsids with varying amounts of granulin deposition were observed. The crystallization of granulin appeared to start most frequently at one end of the virus rod, and half capsules were evi-

dent. Tubular elements (20–30 nm in diameter) were first observed in cells before capsule formation, close to enveloped virus or scattered in areas throughout the cytoplasm (Fig. 7). They differed from microtubules in that they branched, were of variable thickness, and were not hollow appearing. Morphological evidence suggested an association of these tubular elements with granulin formation or transport. That is to say in some sections, deposits of granulinlike material was found among and within these masses of tubular elements (Fig. 8). Mature, occluded virions were associated with islands of coiled tubular elements of the varying diameters (10–25 nm) (Fig. 9). Coiled tubular elements occurred more frequently in infected cells which produced only a few large aberrant capsules or in which capsule formation seemed to be inhibited (Fig. 10). In addition to the coiled tubular elements some capsules were observed to have an associated outer epicapsular layer (Fig. 11). This layer (8–12 nm wide) was separated from the capsule by a fairly uniform distance. The epicapsular layer was not always continuous.

When cells ruptured late in infection releasing their contents into the hemocoel, masses of coiled tubular elements were evident among the cellular debris (Fig. 12). The diameter of coiled tubular elements seemed largest early in infection and de-creased in cells where capsule formation was complete.

The residual components of the nucleus were scattered throughout the cytoplasm (Fig. 3) and appeared to undergo some structural changes. As mentioned before, fragments of the nuclear envelope were observed in the cytoplasm. These fragments were observed singly or in pairs (Figs. 3, 4, 13, 14, 15). Nucleocapsids were sometimes associated with remnants of the nuclear envelope. The nucleocapsids were seen aligned perpendicular to the nuclear pore with the conical or hemispherical end opposed to the pore lumen. In addition, arrays of membranelike complexes (MC) were observed. The MC were composed of continuous sheets of parallel membranelike structures 10 nm in width (Fig. 13). Between the membranelike structures, a fibrillar material was deposited. When cut in cross section, the membranelike complexes appeared as interconnected circular elements and the fibrillar material was sometimes seen as a hollow tubules in the center (Fig. 14). Stacks of these membranelike complexes often had ribosomes associated with the outermost surface (Fig. 18). These were similar to paired cisternae of rough endoplasmic reticulum (RER) that were observed. In the lumen of RER paired cisternae an inner membrane was found (Fig. 15). A close association of these RER cisternae was seen with pieces of nuclear

FIG. 3. In an infected hypodermal cell the granular (G) and fibrillar (F) components of the nucleolus are seen to disperse after the nuclear membrane fragments. Fragments of the nuclear membrane (M) with nucleopores are present as well as cisternae of the rough endoplasmic reticulum with membranelike structures in their lumen (E). × 13,600.

FIG. 4. Enlargement of the boxed area in Fig. 3. This shows the nuclear pores present on remnants of the nuclear membrane. × 68,000.

FIG. 5. Nucleocapsids bud into the hemocoel from a midgut. The nucleocapsids are seen to align themselves under specialized thickened areas of the plasma membrane (the peplomers) where presumably viral envelope proteins have been inserted (arrows). × 60,000.

FIG. 6. In some midgut cells so many nucleocapsids are produced that they align in paracrystalline arrays in the basal regions of the cells. These nucleocapsids possibly represent an overproduction of virus that may not actually bud. Virus which has completed budding accumulates between the plasma membrane and the basal lamina (B). × 30,000.
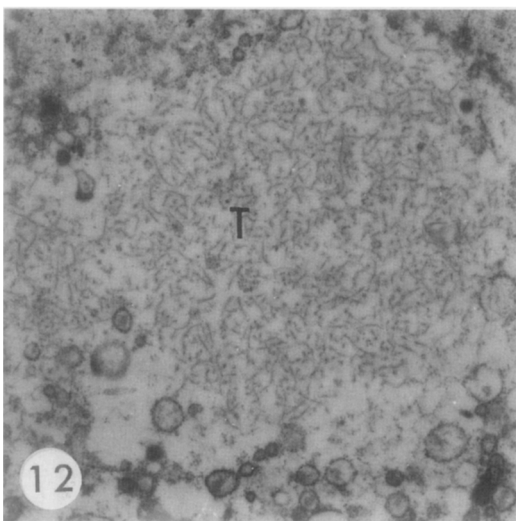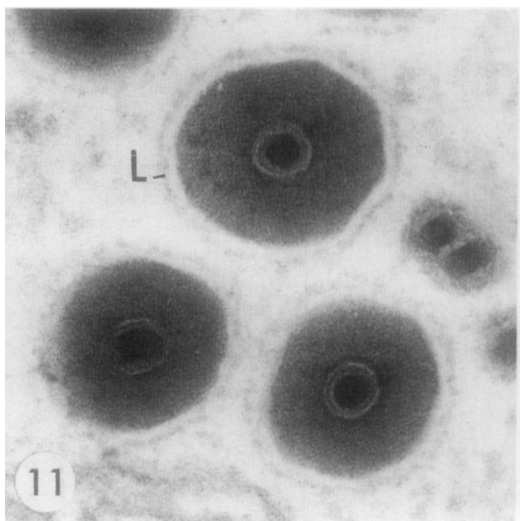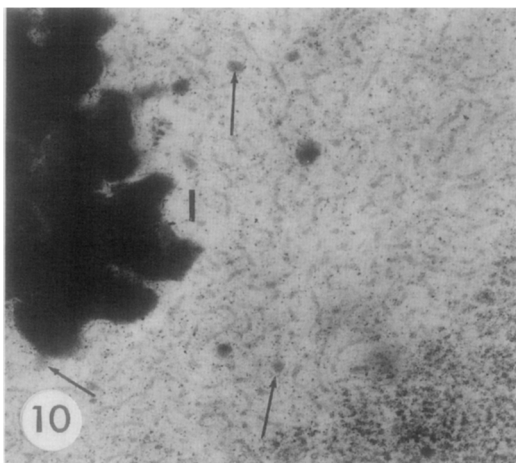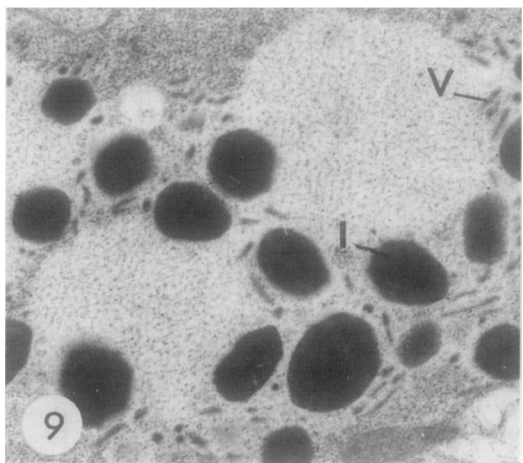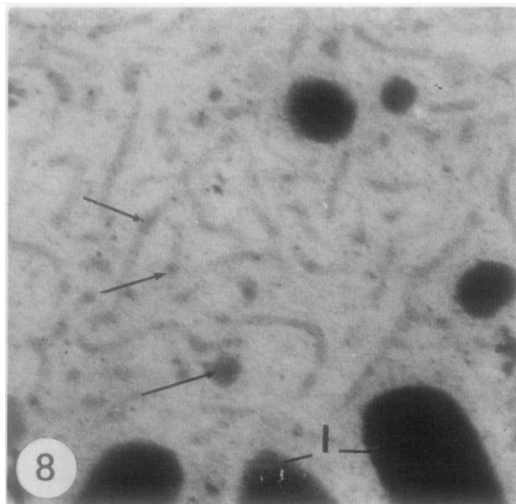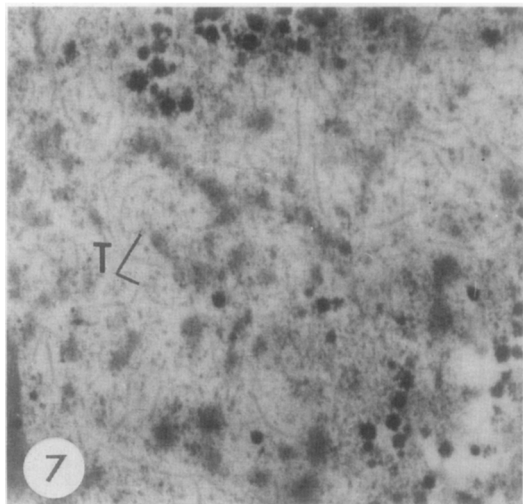
membrane, such as to suggest connection or continuation (Fig. 15). The stacks of MC frequently were observed associated with portions of the granular and dense fibrillar elements of the nucleolus (Figs. 13, 14).

Sections were obtained indicative of the formation of these membranelike complexes among and possibly from the nucleus remnants shown in the following order of assembly: Fig. 1 (not infected), Figs. 2, 3, and 13–18. These sequential steps in the formation of the membranelike complexes were suggested in electron micrographs selected from successive postinfection experimental groups: (1) Within the areas of the fibrillar component of the nucleolus, a decrease in electron density was observed (Fig. 13). The amount of the dense fibrillar component of the nucleolus concomitantly appeared to decrease. Gradations in density between the two areas were observed suggesting that the less dense areas formed from the denser areas. (2) Linear parallel sheets of varying thicknesses were observed within the fibrillar areas (Figs. 13, 14). These sheets were of the same structure as the fibrillar areas. (3) The parallel sheets were observed to increase in numbers while the fibrillar areas declined. The granular components of the nucleolus still were associated with the forming MC. (4) The fibrillar sheets be-came more condensed resulting in the typical 10-nm-thick membranelike structures. Ribosomes were observed associated with the outer surfaces of the MC (Fig. 18). By this time only membranelike structures were present in the cell and the fibrillar and granular components of the nucleolus were no longer evident. In some cases remnants of the nuclear membrane appeared closely associated with the MC and some continuity between the membranes was suggested (Fig. 16). At the same time that the MC were condensing from the fibrillar areas, a structure of similar morphology was observed within the lumen of the rough endoplasmic reticulum (Fig. 17).

Capsules were found at the periphery of the cells sometimes in ordered arrays. As the infection progressed in the cell, cytopathic changes were observed. These changes included a decrease in rough endoplasmic reticulum and loss of normal cellular organelles such as lipid droplets, glycogen, electron-dense granules, multivesicular bodies, and pinocytotic vesicles. The cellular junctions, especially the hemidesmosomes, were no longer apparent. In some cells the mitochondria were observed to be swollen. Eventually cells were observed with breaks in the plasma membrane through which their contents entered the hemolymph.

FIG. 7. Coiled tubular elements (T) were observed in the cytoplasm of fat body cells before capsule formation. These tubular elements differ from microtubules in that they were branched, did not have a central channel, and were variable in diameter along their length. ×28,000.

FIG. 8. The coiled tubular elements appeared to contain a material similar to granulin deposits (arrows). This is not as evident in the mature capsules (I) which are more dense. (Fat body) ×100,000.

FIG. 9. Islands of coiled tubular elements are frequently surrounded by virions (V) and mature capsules (I) in the fat body as well as in other tissues. When only mature capsules are associated with the tubular elements they are of a smaller diameter than when only virions are present. ×33,600.

FIG. 10. Coiled tubular elements are the most evident in cells where few capsules or aberrant capsules (I) are formed such as in this midgut cell. Deposits of granulin-like material are seen in the coiled tubules (arrows). ×40,000.

FIG. 11. Mature capsules are surrounded by an epicapsular layer (L) 8 to 12 nm thick. These are of the same diameter as intermediate filaments. (Fat body) ×126,000.

FIG. 12. When an infected fat body cell ruptures and the contents are released into the hemocoel, islands of coiled tubular elements (T) are evident in the cellular debris. ×11,200.
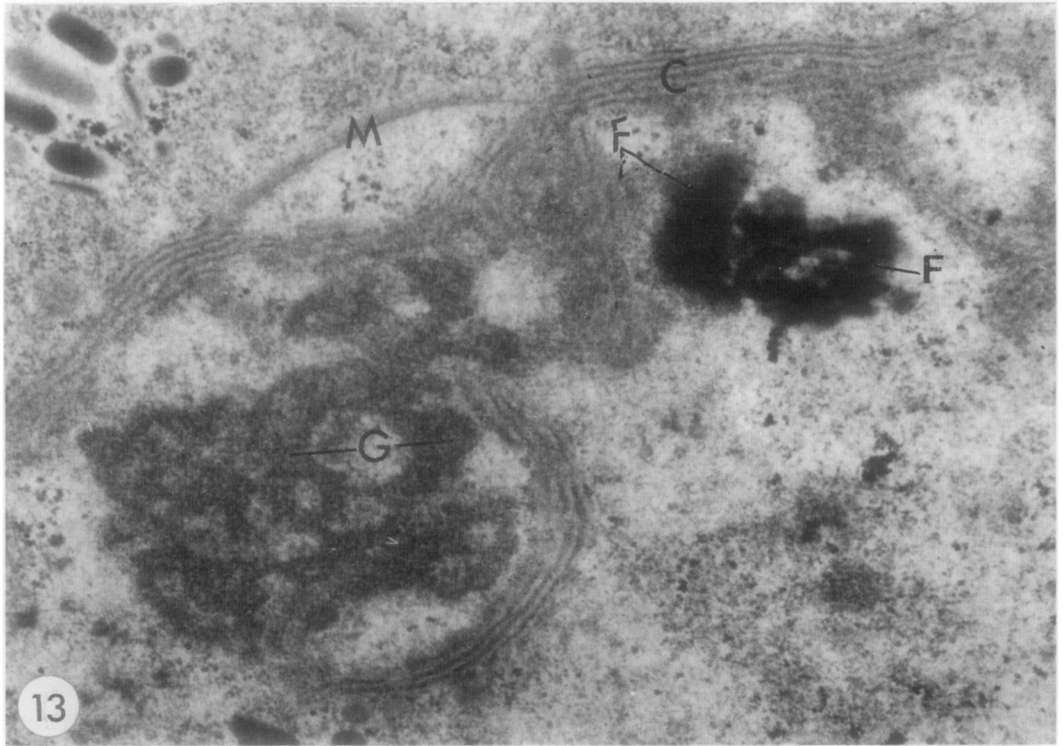
FIG. 13. Membrane-like complexes (C) were seen to form among or possibly from the nucleolar components. Gradations in density between the dense fibrillar components (F) and the substance of the membrane-like complexes are seen. F, granular component of the nucleolus; M, remnant of the nuclear membrane. (Hypodermis) ×56,000.

## Tissue Replication of CMGV

*24 hr postinfection.* The invasion of codling moth tissues by the granulosis virus begins in the midgut. The process of virus uptake was not investigated. At 24 hr postinfection (pi), nucleocapsids were already observed passing through the basal and basolateral plasma membrane into the hemocoel. The virus was not occluded in the midgut nor were any stages suggestive of encapsulation, i.e., the formation of coiled tubular elements or the accumulation of deposits of granulin, observed at 24 hr pi. The midgut cells exhibit a polarity in virus uptake, development, and movement generally consistent with its functional aspects. Developing virus was observed in the areas where the nucleus once was present. Nucleocapsids moved basally and laterally to

bud through the plasma membrane. There was a thickening of the plasma membrane associated with budding into intercellular spaces as well as along the basal surfaces of the cell (Figs. 5, 6, 19). Budding resulted in the accumulation of enveloped virions in the intercellular space as far up as the midportion of the cell (Fig. 19). In many instances the accumulation of virus in the infoldings of the plasma membrane gave the appearance of the virus being within vacuoles (Fig. 19).

Basally the virions accumulated most frequently in the intercellular space (Fig. 19) and less frequently in the space between the basal plasma membrane and the basal lamina (Figs. 6, 19). Virions were already present in the enclosing muscle and tracheal tissues. Virions were within the cytoplasm of these cells as well as between

the layers of basal lamina investing them. Virus movement in midgut cells was not observed into the apical-lateral regions of the plasma membrane where extensive cell junctions were observed or into microvilli. In some infected cells, there was a considerable accumulation of nucleocapsids in paracrystalline arrays at the plasma membrane preparatory to budding (Fig. 6). In the cells with extensive viral replication, some of the membranelike complexes associated with nuclear remnants were beginning to form.

Columnar cells as well as endocrine cells were infected. Goblet cells were not yet infected. In the basal regions of the midgut, regenerative cells were observed with many nucleocapsids within the nucleus. These cells did not exhibit nuclear "clearing"; however, it is possible that either they had previously been infected and these represented replicating virus or they had just been invaded by the virus and these nucleocapsids represented invasive forms.

Despite the enormous amount of budded virus produced by the midgut at this time, efforts to locate any invasive virus associated with the cells of the fat body or hypodermis were futile.

In the fat body at 24 hr some of the nuclei were observed to be in various stages of clearing. In a few other nuclei nucleocapsid formation was already observed. The nuclear envelope of most infected fat body cells was intact. Some increase in nuclear pores appeared to have occurred. The hypodermis at 24 hr pi showed no signs of virus replication even when closely associated fat body nuclei contained virus. This was true even in hypodermal cells whose basal surface was open to the hemocoel.

*32 hr postinfection.* The most obvious change in the midgut at 32 hr pi was the production of capsules. At this point all stages of typical virus replication were observed. In the basal and basolateral regions of the cells, active virus budding continued but was diminished from that seen at 24 hr.

In many cases budding was limited to cells which had not begun to produce capsules or in which capsule production seemed to be inhibited. In these cells, the islands of coiled tubular elements were very apparent. Signs of abnormal capsule formation observed at 32 hr pi in the midgut included the production of irregularly shaped capsules and large cuboidal capsules. In addition, some cells in the midgut of a few insects were observed to envelope and occlude multiple virions in a manner similar to nuclear polyhedrosis viruses (Falcon and Hess, 1985). In most cells, however, virus replication and occlusion was observed to be normal. Infected goblet cells were also observed.

Some cytopathic changes were observed within midgut cells at this point. In many cells, small cuboidal crystals were observed within the dilated rough endoplasmic reticulum. Although dense lamellar bodies were observed in some control midgut cells, the frequency in infected cell had increased at 32 hr pi. In addition, some mitochondria were swollen while others were observed to form doughnut shapes. Electron-dense granules containing virus, possibly lysosomes were observed. Some large cellular vacuoles contained sequestered organelles as well as electron-dense membranes.

In the fat body by 32 hr pi, the majority of the budding had occurred and substantial numbers of enveloped virions accumulated between the plasma membrane and the basal lamina. In some cells so many virons were present that the basal lamina was lifted away from the cells and the hemidesmosomes were disrupted or no longer present. Budding appeared to occur predominately on the surfaces of the fat body cells covered by the basal lamina. Some groups of budded virus occurred in the intercellular spaces; however, junctions were observed on the membranes on either side of the virions in the plane of sectioning. All stages of normal virus replication were observed and many cells were already packed
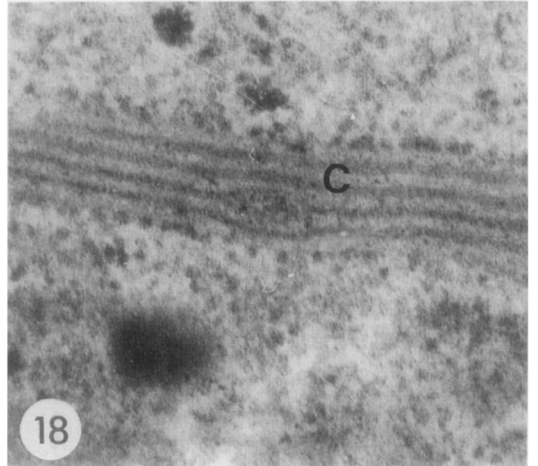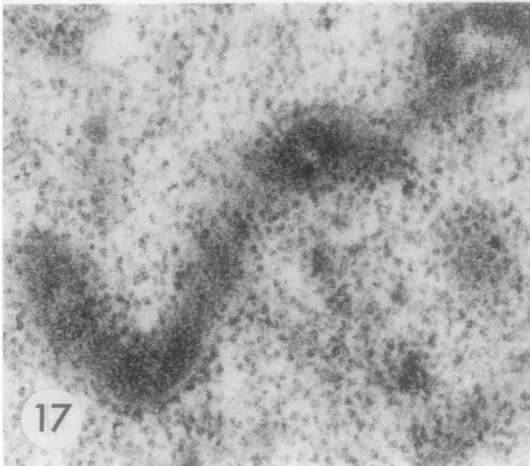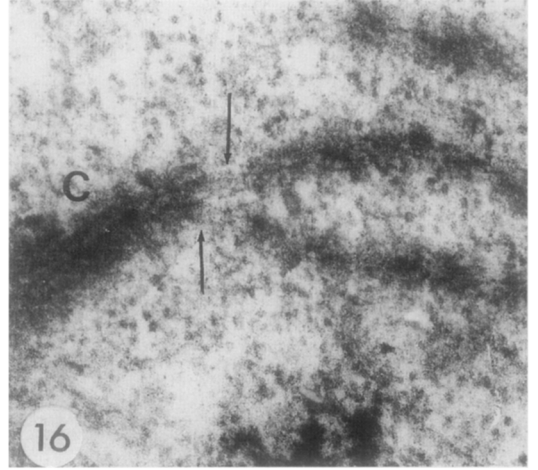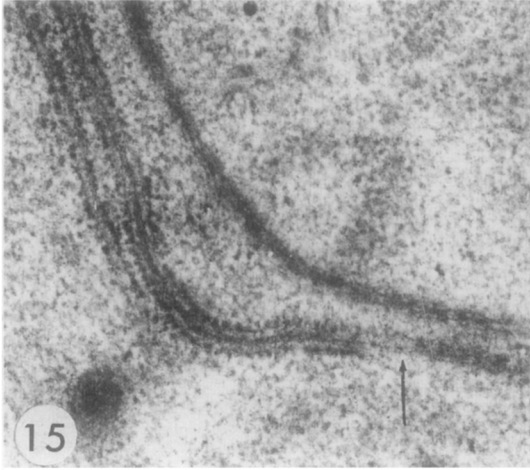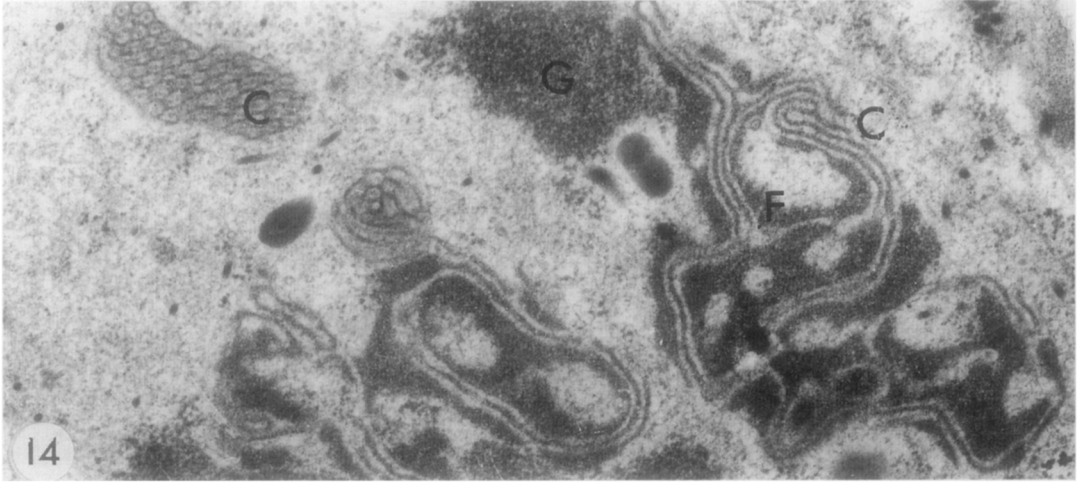
with capsules. In these cells a decrease in lipid droplets as well as a depletion in glycogen were observed. Few cells were not infected. Some infected cells were observed which were disrupted and contained breaks in the plasma membrane. The contents of these cells were held from entering the hemolymph by the intact basal lamina.

Healthy appearing cells alternated with infected cells in the hypodermis at 32 hr pi. As in the fat body, all stages of viral replication were present. Many cells contained capsules. In general, infected cells had accumulated budded virus in the space between the basal lamina and the basal plasma membrane. As seen in the fat body, this was accompanied by the disappearance of hemidesmosomes along the basal regions of the cell. Intercellular junctions appeared to be intact. Virus budding was observed only from the basal surface of the cells. Capsules formation, however, was observed around the entire perimeter of the cell, indicating that nucleocapsids moved in all directions.

The electron-dense granules found in normal epidermal cells were not observed in infected cells or in the immediately surrounding uninfected cells. Some granules were observed in large groups of uninfected cells particularly those not directly exposed to the hemocoel because of

closely opposed muscle cells. In some infected cells, a change in the cuticle–epidermal cell association was observed. Microvilli were sparse, shortened, and even absent. In some cases large gaps between the epidermis and the cuticle were present. This was particularly true when the epidermal cells were heavily infected. In most cells the basal glycogen deposits appeared similar as those in the controls.

*48 hr postinfection.* At 48 hr pi the midgut still had a large population of uninfected cells compared to the fat body. Virus budding was continuing in some cells. In many other cells a large number of nucleocapsids had accumulated in the basal portion of the cells and many budded viruses had collected within the intercellular spaces. However, the amount of budded virus in the surrounding muscle and tracheal cells had diminished significantly from 24 hr pi. In the virogenic areas of some cells abnormally long nucleocapsids were observed. In many cells nucleocapsid assembly had apparently ceased and few nucleocapsids were to be seen in the central virogenic area of the cell. Some cells were observed with many cuboidal crystals within the rough endoplasmic reticulum. In these cells, capsule production was absent or severely depressed. In other cells, some groups of nucleocapsids were located

FIG. 14. Membrane-like complexes (C) were composed of linear parallel sheets that appeared among the less dense fibrillar (F) areas putatively formed from a disassociation of the dense fibrillar components of the nucleus. In cross section they appeared as interconnected circular elements. G, granular component of the nucleolus. (Fat body) ×28,125.

FIG. 15. The cisternae of the rough endoplasmic reticulum in infected cells frequently contained a membrane-like structure within their lumen. These membrane-like structures in the midgut were apparently continuous at points with the one of the membranes of the nuclear envelope remnants (arrow). ×43,750.

FIG. 16. The membrane-like complexes (C) also showed areas of apparent continuity between them and pieces of the nuclear envelope (arrows). (Fat body) ×50,000.

FIG. 17. Early in infection some cisternae of the rough endoplasmic reticulum contained in their lumen a substance of similar structure as the less dense fibrillar areas from which membrane-like complexes formed. (Midgut) ×60,000.

FIG. 18. In their final form, the membrane-like complexes (C) such as these in the hypodermis are seen as stacks of parallel membrane-like structures. The outer surfaces of these stacks have ribosomes associated with them. ×86,800.
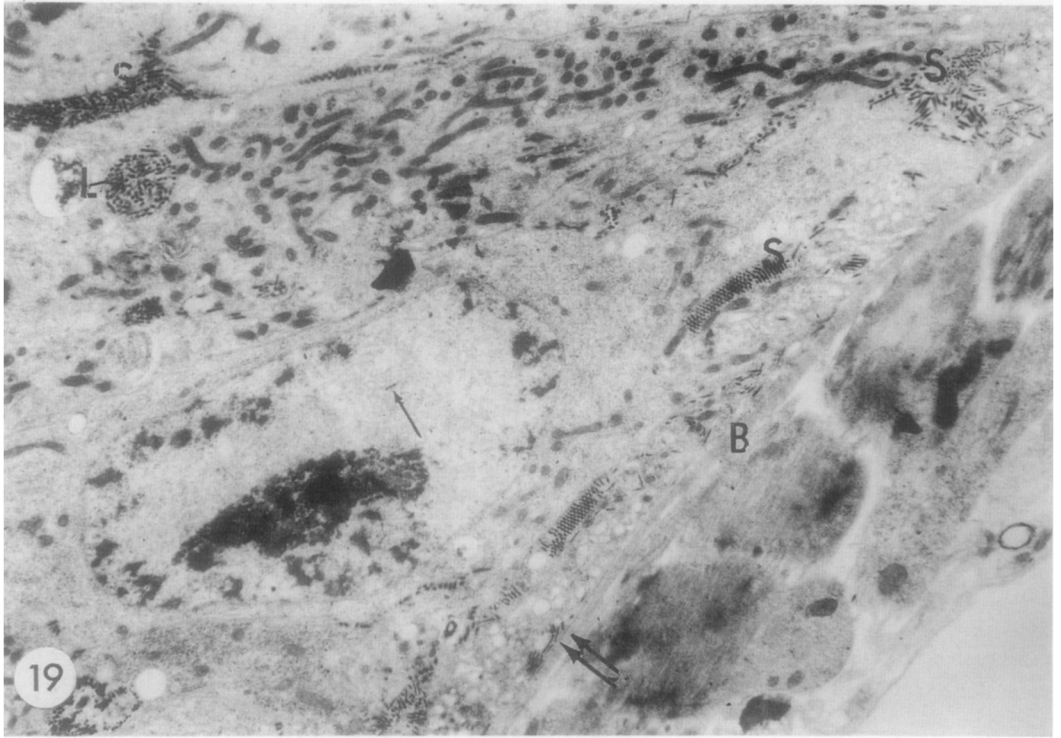
FIG. 19. Cell polarity is evident in this infected midgut cell. Virions have budded into the basolateral and basal intercellular spaces (S). In some cases infoldings of the lateral plasma membrane appeared as vacuoles (L). A nucleus which has not cleared contains a partially empty nucleocapsid (arrow) which may represent an uncoating invasive virion or progeny virus. Virions which have budded (double arrow) are held from entering the hemocoel by the basal lamina (B). ×9,800.

within electron-dense deposits both intracellularly and within the intercellular space.

At this time in the infection, the vast majority of fat body cells had been infected and had produced capsules. No signs indicative of budding were observed and the amount of budded virus held between the plasma membrane and the basal lamina had significantly decreased. Within the hemocoel, capsules, enveloped and unenveloped nucleocapsids, as well as cellular debris were observed, indicating that disruptions in the basal lamina had occurred. Blood cells appeared frequently associated with degenerating fat body cells. In virogenic areas of the infected cells, few nucleocapsids were apparent. Some lipid and glycogen deposits were still present in cells.

In the epidermis at 48 hr pi, there was still a population of uninfected cells. Cells in all stages of virus replication were observed, although earlier infected cells had few nucleocapsids in their virogenic area and were packed with capsules. Unlike the fat body, however, no cells with ruptured membranes were observed. The space between the basal plasma membrane and the basal lamina remained packed with budded virions even in the cells where budding no longer was occurring. The intercellular junctions were intact although the basal hemidesmosomes were reduced or absent in some cells.

*56 hr postinfection.* At 56 hr pi the number of infected cells in the midgut had increased and all stages of viral replication could be found. This still included actively budding virus. In some infected cells, concentric whorls of rough endoplasmic retic-

ulum were observed. Similar whorls were not obvious in all midgut cells and were not seen in infected fat body or epidermal cells. The rough endoplasmic reticulum lumen was filled with a fine fibrillar deposit (Fig. 17). Degenerating infected cells were observed pushing into the gut lumen. Many cells were found in which the plasma membrane had ruptured spilling the cellular contents, including virus, into the gut lumen. The intercellular junctions attaching these cells to their neighbors had degenerated. However, cells immediately beneath or adjacent to those in the lumen were interconnected, indicating that movement of neighboring cells into gaps and subsequent formation of intercellular junctions may be quite rapid.

The fat body now contained many degenerating cells. Other fat body cells filled with capsules had a very clear cytoplasm with few recognizable organelles present. These cells contained vacuoles with electron-dense membranous lamellae, swollen and partially disrupted mitochondria, and lipid droplets. Blood cells were associated with the surface of degenerating fat body cells as well as hypodermal cells particularly at breaks in the basal lamina. The hemocytes were also observed engulfing cellular debris in the hemocoel. Most cells in the fat body had terminated virus production and most virions were encapsulated.

At 56 hr pi, many of the cells of the epidermis had also completed viral replication and contained many capsules. In these cells extensive detachment from the cuticle was observed and the innermost cuticular lamellae were disrupted. In other areas the apical microvilli were absent or, if present, reduced in size and numbers. The characteristic attachment plaques between the tips of the microvilli and the cuticle were not present. In some epidermal cells intercellular junctions were absent and the intercellular spaces were swollen. Some cellular debris as well as budded virions were observed within these spaces as well as in the regions between the basal portion of

the cell and the basal lamina. The basal lamina was observed to have virions within its matrix indicating a possible loosening of the substructure.

*72 hr postinfection.* Most of the larvae examined at 72 hr pi were moribund. Examination of their tissues revealed a range in the cytopathology, indicating that the final stages in cellular death may occur quite rapidly. The midgut showed little qualitative changes at the electron microscope level from the condition at 56 hr. Uninfected cells as well as degenerating cells could be found. Some abnormalities in the virus' replication could be seen. Nuclei were observed in which the nuclear membrane was apparently unable to disassemble. These nuclei were characterized by a large population of intranuclear nucleocapsids. They were not enveloped but accumulated in such quantities as to be regularly packed in paracrystalline arrays. The nuclei were different from control nuclei and normally infected nuclei in that the inner fibrous lamina with its associated chromatin was very prominent. In other cells very long nucleocapsids were found as well tubular profiles which possibly represent defective or excessive capsid formation. The numbers of budded virus observed held between the basal lamina and the midgut cells had decreased. The structure of the basal lamina was more diffuse and discontinuities were present.

Whereas in some insects the fat body was much the same as at 56 hr pi, in other insects the fat body appeared to be almost entirely disassociated. Some islands of intact cells were observed in the hemocoel among the remnants of cells. Uninfected nuclei were also observed. On the exterior plasma membrane of these cells, clumps of enveloped virions were attached.

The hypodermis showed the most variability in infective stages present at 72 hr pi. In the more extreme cases the epidermis was completely detached from the cuticle and the cuticle itself was represented by only the epicuticle and exocu-

ticle. In other cases the cuticle was present; however, the lamellae of the endocuticle appeared to be disintegrating. In areas where the cuticle maintained its integrity, disrupted cells had pushed up between intact epidermal cells into the space under the cuticle. This space was then filled with capsules and cellular debris. In some insects the epidermis was essentially unchanged from that observed at 56 hr pi. In fact, some epidermal cells were not infected at all. Many other cells, although filled with capsules, did not show any signs of lysis and even retained stores of glycogen. Many uninfected cells and those cells which were not very advanced in infection appeared to be protected from the hemocoel by surrounding layers of muscle and tracheal and neural cells, as well as by some residual cells from the fat body. However, others were observed without these protective cells.

Other infection-free cells were observed in moribund insects. Although the tracheal system was not included in this study, it was noted that trachea seemed to be resistant to infection by this granulosis virus. Infected tracheal cells were observed easily after 32 hr pi; however, uninfected tracheal cells were very abundant even at 72 hr pi. In addition, no infected hemocytes were seen in this study. At 56 hr pi hemocytes were abundant throughout the fat body and epidermal layers. These hemocytes contained many sequestered capsules and virions within vacuoles but no signs of viral replication were observed.

In some infected cells, unusually long arrays of fibrous strands similar in morphology to condensed chromosomes were observed. These cells did not have the residual nuclear components in the cytoplasm reported present in the normal GV replication. Since they occurred only infrequently it was not possible to follow the developmental sequence.

## DISCUSSION

The basic replicative sequence observed with the electron microscope for CMGV is fairly similar to that observed with other granulosis viruses (Walker et al., 1982; Pinnock and Hess, 1978; Tanada and Leutenegger, 1970; Granados, 1980; Summers, 1971). Some exceptions, however, do occur.

In the codling moth, the first overt sign of infection is a clearing of the nucleus. Viral entry into the nucleus was not observed, although nucleocapsids were observed in nuclei where clearing had not occurred. In these cases it was impossible to determine whether these nucleocapsids represented invading virions or viral progeny. The site of entry of granulosis virus into the nucleus has been suggested by electron microscope studies to be through extranuclear uncoating at the nucleopores (Summers, 1971; Granados, 1980). Walker et al. (1982) observed intranuclear nucleocapsidlike structures associated with the nucleopores 1 day posttreatment that are similar to those observed in this study in that the exact origin cannot be determined. Occasionally nucleocapsids were observed associated with the pores on remnants of the nuclear envelope. This association, seen late in infection, suggested some preferential alignment of nucleocapsids with nucleopores. Whether these nucleocapsids represent invasive virus stuck on the outside of a nucleopore early in infection and unable to uncoat cannot be determined. They could also represent nucleocapsids which associated with the nucleopores within the nucleus prior to fragmentation of the nuclear envelope or nucleocapsids formed much later in infection which then encountered the remnants of the nuclear membrane. This association could be simply related to a charge affinity or may indicate attachment sites utilized by the virus possibly during uncoating. Walker et al. (1982) reported small intranuclear blebs of the inner nuclear membrane as the first recognizable alterations in the granulosis-infected cells of *Spodoptera frugiperda*. These were not observed in the codling moth. Intranuclear annulate lamellae and crystalline deposits on the nuclear

membrane reported in other GV infections (Pinnock and Hess, 1978; Walker et al., 1982) were also not observed. An increase in nuclear pores in infected nuclei prior to nuclear envelope disassociation was found in the codling moth. A similar increase in nuclear pores was noted by Walker et al. (1982) in *S. frugiperda*.

In the course of infection, many membranous and tubular arrays were observed. Concentric whorls of rough endoplasmic reticulum were obvious in the midgut of the codling moth. In some granulosis infections, a decrease in RER has been reported. Indeed, a reduction was noted in most cells other than in the midgut in this study as infection progressed. These RER whorls were most obvious in the midgut cells in which an excess of budded virus was produced over a long period. They persisted throughout the infection. Since envelope glycoproteins of budded virus are probably synthesized in the RER very early in infection, probably before and during the appearance of the first nucleocapsids, the importance of the RER in granulosis virus replication cannot be overlooked. Envelope proteins are probably inserted at the thickened specialized regions of the plasma membrane through which baculoviruses bud (Volkman et al., 1984). Midgut cells which produced budded virus throughout the course of infection, in contrast to fat body and epidermis, would be expected to maintain RER longer in infected cells. An abundance of RER has also been noted in, for example, the midgut infection of *Trichoplusia ni* (Tanada and Leutenegger, 1970).

Many events in the replication of CMGV appear to parallel events normally observed in a dividing cell. In prophase the nuclear membrane is seen to break down and the nucleolus disassociates into as many as 10 individual fragments. Nuclear envelope reformation and nucleolus reassociation subsequently occur at telophase. The CMGV replication apparently either mimics, induces, or fortuitously utilizes this phase of the cell cycle to its own ben-

efit thereby allowing the virus unrestricted access to the cytoplasmic and nuclear components of the cell for synthesis and assembly of progeny. This is in contrast to the nuclear polyhedrosis viruses where assembly is in most cases restricted to the nucleus and proteins must be transported from the cytoplasm for utilization. It has been previously suggested that CMGV and other baculoviruses may initially stimulate cell division (Benz and Wäger, 1971; Morgante et al., 1974). Benz and Wäger (1971) concluded that infection in a cell induces all those biochemical processes which eventually could lead to cell division, although no mitoses were observed in CMGV-infected fat body. An increase in mitosis has, however, been observed with some granulosis infections (Wittig, 1959; Hamm, 1968; Walker et al., 1982).

In cell division, after the nucleolus enlarges and divides into multiple fragments, it disappears until telophase. In CMGV infection the pieces of the nucleolus disperse but remain visible with the electron microscope. These are seen as fibrillar and granular components. Later in infection the formation of membranelike complexes was associated with the nucleolus remnants. Wäger and Benz (1971) described pseudonucleoli associated with CMGV infection. These small pyroninophilic bodies stain at approximately the same time that the membranelike complexes are seen to form within the residual nucleoli with the electron microscope. They contain RNA (Benz and Wäger, 1971) and it was suggested that they are synthesized de novo or represent aggregations of ribosomes derived from the former nucleolus. The fragmentation of the nucleolus has been observed with other baculovirus infections. In the case of *Rhynchosciara angelae* infected with a nuclear polyhedrosis virus, a dramatic increase in RNA synthesis is accompanied by increased size of the nucleoli (Morgante et al., 1974). The main nucleolar body was seen to fragment off pieces of smaller nucleoli. In other cases the intact nucleolus has been observed to persist throughout

granulosis virus infection (Pinnock and Hess, 1978).

In this study, electron micrographs suggested that the membranelike complexes may be formed from or within the fibrillar component of the nucleolus. It is within this component that RNA transcripts are normally believed to be located. The granular component of the nucleolus, containing the most mature of the ribosomal precursor particles, was also closely associated with the forming membranelike complexes. The nature of the membranelike complexes has yet to be resolved. They were similar in structure to the paired cisternae of rough endoplasmic reticulum that were also observed. It was seen that the cisternae of the rough endoplasmic reticulum contained membranelike profiles in their lumen which were apparently continuous with fragments of the nuclear envelope. Similarly, MC which had ribosomes on their membranes were also seen to be continuous with pieces of the nuclear envelope. Membranelike complexes certainly seem to occur quite frequently in granulosis virus infections. In an electron microscope study of CMGV infections, Tanada and Leutenegger (1968) referred to them as coiled membranous structures surrounded by electron-dense material. They suggested that they might represent nuclear fragments. In the potato tuberworm, *Phthorimaea operculella,* the MC were called remnants of the nuclear membrane which appeared in myelinlike whorls (Hunter et al., 1975). In *Archips argyrospila* the equivalent structures were considered stacks of annulate lamellae (Pinnock and Hess, 1978). Walker et al. (1982) called them arrays of tubular membranes and suggested that they may originate from paired cisternae or from excessive production of cellular membranes.

The membrane complexes and paired cisternae of the RER seen in this study appear to be structurally similar to laminated cisternae of the RER induced during infection of murine fibroblasts by a coronavirus, mouse hepatitis virus strain A59 (MHV-A59) (Tooze et al., 1985). In MHV-A59 infections the viral structural glycoproteins $E_1$ and $E_2$ were shown to be present in the membranes of the paired RER cisternae. It was suggested that the pairing resulted from an alteration of the normal budding mechanism in which the $E_1$ molecules projecting from the faces of two adjacent cisternae associate with the same viral nucleocapsid in the cytosol thereby causing pairing to occur. The surfaces of the RER cisternae were not strictly polarized and pairing could occur on either surface resulting in stacks, although three cisternae laminated together were seen only rarely. Whether a similar mechanism may be operating in CMGV is at this point only speculative but seems reasonable.

A difference in the polarity of the cells in the midgut, fat body, and epidermis was observed in this study in respect to virus invasion and the subsequent release of virus progeny. In the midgut, the primary invasive virus is known to enter at the microvilli via fusion at the plasma membrane. Budding of progeny, secondarily invasive, virus was observed to occur in the basal-lateral and basal regions of the cells. Most of the budding appeared to occur into the intercellular spaces where cellular junctions were absent. Passage of the virus across the apical regions of the plasma membrane was not observed. In the fat body and hypodermis the initial site of infection was not observed. Entry into these and other tissues by the budded virus in the hemocoel is probably predominately by viropexis (receptor-mediated endocytosis) and to a lesser extent by fusion as seen by others (Tanada et al., 1984; Adams et al., 1977). With the epidermis, only the basal regions of the cell are exposed to the hemocoel and it would appear that this would be the site of virus entry. Release of the virus was only observed along the basal regions of the epidermal cells. Accumulation of large amount of virus between the basal plasma membrane and the basal lamina was

observed to be associated with a disruption of the hemidesmosomes which attach the basal lamina to the cell. The epidermal cells appeared to have junctions along the entire lateral plasma membrane and no virus was observed in the intercellular space until late in infection when the intercellular junctions were disrupted. Budded virus was never seen in the apical portion of the cell beneath the cuticle. In the case of the fat body, virus could potentially enter from any surface open to the hemocoel. Virus was observed to bud from any surface of the fat body cells. The preferential point of budding appeared to be those areas of the plasma membrane open to the hemocoel; however, virus was observed to also exit into intercellular spaces where junctions were not present. The nuclear polyhedrosis virus of *Autographa californica* is known to insert a viral glycoprotein into the plasma membrane in vitro. The progeny virus buds through the altered membrane and acquires its envelope with associated viral glycoprotein (Volkman et al., 1984). In vivo budding of virus does not occur in regions of the cell membrane where specialized junctions occur and in the case of the epidermis and midgut does not occur from the apical regions of the cells. This would indicate that there may be distinct domains on the plasma membrane where viral glycoproteins may not or cannot be inserted and therefore through which budding cannot proceed. In the case of the epidermis and midgut nucleocapsid, movement during viral release was also predominately toward the basal regions where these glycoproteins were probably present.

The basal lamina seemed to represent a barrier to the full release of budded virus into the hemocoel and it appeared that budded virus may be actually overproduced as a result. As infection progressed it was observed that there was a continuous production of budded virus from the midgut cells but in the epidermis and fat body budded virus was produced early in infection, accumulated beneath the basal lamina, and then gradually diminished until few were observed. How the virus passes through the basal lamina is unknown. The basal lamina forms a continuous layers over all the cells bordering the hemocoel except for hemocytes. At 24 hr postinfection, many virions were observed in the muscle and tracheal tissue basal to the midgut and virus replication was already evident in the fat body. However, very few micrographs were obtained of virions within the substance of the basal lamina and this was not until late in infection. In an NPV infection of tracheal cells the virus was observed to move laterally along the basal lamina to infect adjacent cell (Tanada et al., 1984). The basal lamina is produced and secreted by the underlying cells. In the beginning of the fourth and fifth stadia and presumably at other times, it has been reported that the fat body phagocytoses its basal lamina (Locke, 1984). The hemidesmosomes concentrate in an area that ultimately invaginates to become a phagocytic vacuole pulling with it the basal lamina. In *Manduca sexta* the basal lamina of the epidermis is seen to thin on days 4, 8, 9 of the 5th larval instar (Sedlak and Gilbert, 1979). Since codling moth larvae are most susceptible to virus infection during times that the basal lamina may be the thinnest (Etzel, 1973) it would appear to be a significant factor in virus spread.

The actual movement of CMGV throughout the tissues of the larval host is governed by sequential cycles of virus invasion, replication, and release. The midgut was seen to play a key role in the release of invasive progeny in that capsule production is delayed in this tissue, budded virus is produced over a long period of time, and the infection of adjacent midgut cells proceeds slowly. Even when larva were moribund cells were observed in the midgut in the early stages of infection. This is in contrast to the fat body where infection progressed quite rapidly and by 48 hr postinfection most cell were infected and

had produced capsules. Degenerative fat body cells were easily observed and capsules found in the hemocoel all apparently originated from the fat body. Epidermal cells were slower to become infected than fat body cells. At 24 hr postinfection, some fat body cells were already producing nucleocapsids, but adjacent epidermal cells showed no signs of infection. As in the midgut, the spread of the infection throughout the epidermis also was considerably slower than in the fat body. The characteristic melting of the larvae apparently is caused by the degradation of the inner layers of the cuticle and the lysis of infected cells below. Some areas of the epidermis were afforded protection from infection by investing layers of uninfected muscle and trachea cells. Although subject to infection, many tracheal cells were also observed not to be infected after 72 hr. Residual cells were seen in all tissues that appeared resistant to infection. These cells may be portions of the cell population in which cell death has already been programmed so that the virus could not switch on the appropriate mechanisms for either invasion or replication. Interestingly, it was observed that these cells had virus adhering to the exterior of their plasma membrane possibly indicating that virus may not have been able to invade these cells after attachment.

These tissue-associated differences in virus replication times are no doubt related to gross differences in metabolism as well as population differences in functional types of cells as well as age. The fat body in many insects is a uniform tissue with only one cell type, the trophocyte. This cell is primarily concerned with the synthesis and secretion of proteins, lipid and glycogen metabolism, and the storage and breakdown of these products. In contrast, the midgut contains a diverse cell population of which columnar cells appear the most susceptible to infection. Even within the columnar cell population, however, a great diversity in functions occurs and this appears to be reflected in the time spread it

took for individual cells to eventually begin producing virus.

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