ON THE MULTIPLICATION OF AN INSECT VIRUS*

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One type of virus affecting insects is the polyhedral virus. It is characterized by the formation, within the nuclei of susceptible cells, of protein crystal-like bodies (polyhedra) from less than one to more than 10 microns in diameter. BERGOLD[3] demonstrated that a polyhedron consists of about 95% by weight of non-infectious protein and about five per cent of infectious virus particles. Polyhedral bodies dissolve in weak alkali and the virus particles which they contain are liberated. These can be separated from the non-infectious polyhedral protein by centrifugation. The particles isolated in this manner are highly infectious. Fifty per cent of silkworm larvae injected with about 10^{-13} g of virus died from disease[4].

Electron micrographs of purified virus preparations show a large number of rod-shaped particles and frequently also spherical particles. The rods have dimensions of about 30 to 50 mμ in diameter and 260 to 360 mμ in length, depending on the insect species[3,4,6,18]. The spheres are assumed to be early stages in the development of the rods. According to BERGOLD[5], the virus appears as a minute spherical body. This body increases in size and the rod-shaped particle appears as an elongated, curved body, surrounded by a membrane. Later the rod straightens out, ruptures the membrane, and escapes leaving an empty spherical membrane behind. A lengthwise groove is sometimes observed in the developing rod which indicates that two rods may develop from one sphere. Frequently empty tubular-shaped membranes are observed indicating that the rods are enclosed by a second membrane and that the naked rod-shaped particle escapes when this second membrane ruptures.

Since the starting material for obtaining virus preparations is always purified polyhedra, there is no doubt that the particles observed were contained within the polyhedra. Electron micrographs of partially dissolved polyhedra have shown many bundles of rod-shaped particles in one polyhedron[6,12]. It is assumed that the virus multiplies in the free state in the cell and later protein material produced by the cell surrounds the particles to form the crystalline polyhedra. The term "occlusion" is frequently used to describe this process. A process such as this would explain the presence of many of the stages of virus development within polyhedra.

Polyhedral virus diseases of insects have been recognized and studied for many years. These studies have been restricted to infections of insects in the Order Lepidoptera

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and chiefly to the diseases of the silkworm, *Bombyx mori* L., the nun-moth, *Lymantria monacha* L., and the gipsy-moth, *Porthetria dispar* L. It is generally agreed that, in the larvae of these insects, polyhedral bodies are formed in the nuclei of the tracheal matrix, hypodermis, fat, and blood cells. Studies by BREINDL[9] indicate that all tissues except the gonads, Malpighian tubules, and alimentary tract eventually become infected with virus.

The nuclear alterations associated with virus invasion and polyhedral formation have been many times described[10,11,13,14,15]. The first symptom of infection, observed in infected blood cells under the dark-field microscope, is a ring zone of minute bluish refracting granules between a dark central mass and the nuclear membrane. Later these granules increase in size and appear as polyhedra. Studies of sectioned and stained tissues show that the dark central mass observed under the dark-field microscope consists of coagulated chromatin. LETJE[14] observed that, during the disintegration of the nucleus, the chromatin loses its pearl-bead-like structure, becomes somewhat thickened, and undergoes chromatolysis (pycnosis). The chromatin constricts more and more to a single homogeneous central mass in the nucleus and its granular structure disappears completely. The polyhedra arise as small granules in a ring zone between the chromatic mass and the nuclear membrane. KOMAREK AND BREINDL[13] and PAILLOT[15] observed the formation of polyhedra within the chromatic masses. GLASER[10] observed that the chromatic mass persists at times in the polyhedra-filled nucleus, but more often it disappears.

The European spruce sawfly, *Gilpinia hercyniae* (Htg.), an insect in the Order Hymenoptera, is susceptible to a polyhedral virus disease[1,8]. The disease differs from similar infections of lepidopterous insects chiefly in that polyhedral bodies are formed only in the nuclei of the digestive cells of the mid-gut epithelium; the tracheal matrix, hypodermis, fat, blood cells, cells of the fore-, and hind-guts, and other tissues are not susceptible to infection.

This paper describes the formation of polyhedra within the nuclei of infected cells of European spruce sawfly larvae, and particles isolated from polyhedra. The origin of particles within polyhedra, based on an electron microscope study of thin sections and material extracted from infected cells, is also described.

METHODS

Light microscopy

Normal cells and cells in various stages of virus infection were studied under the dark-field microscope. Small pieces of the gut wall were removed and flattened between slide and cover slip.

Normal and infected tissues were fixed in BOUIN'S fluid, cut at four microns in thickness, and stained with HEIDENHAIN'S iron haematoxylin. The chromatin of the nucleus was identified in tissues fixed in ZENKER'S fluid and stained by FEULGEN'S method.

Electron Microscopy

Virus purification

Purified polyhedra were dissolved in weak alkali (0.007 *M* Na_2CO_3 and 0.05 *M* NaCl). After three hours the suspension was centrifuged in a Sorvall angle centrifuge at 6,500 r.p.m. for five minutes to remove polyhedra not completely dissolved. The supernatant was centrifuged at 11,700 r.p.m. for one hour. The resulting supernatant was discarded and the pellet was suspended in distilled water. This suspension was centrifuged at 6,500 r.p.m. for five minutes and the supernatant was examined under the electron microscope for virus particles.

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Extraction of particles from living cells

Particles were extracted from living cells in the following manner. Alimentary tracts of infected insects were removed and inflated with air. The inflated guts were pierced with fine glass tubes. Some of the cell contents was drawn into the tubes through capillary action. The material drawn into the tubes was diluted with distilled water to the desired concentration and placed on screens for examination under the electron microscope.

Preparation of thin sections

Thin sections of normal and infected tissues were prepared using a technique similar to that described by PEASE AND BAKER[16].

The alimentary tract of a larva is removed, inflated with air, and fixed in modified KAHLE's fluid[17] while inflated. After a few minutes the alimentary tract becomes rigid and is then sliced into small squares. The small blocks of tissue (approximately 0.5 mm thick and 1 mm square) are placed in the fixative for two hours, run up through alcohol series into ether-alcohol, then into three, five, and finally 12% collodion dissolved in ether-alcohol. The collodion is hardened in chloroform, and the blocks are transferred to xylol by way of carbo-xylol. They are then infiltrated with 65° C paraffin.

The sections are cut on a Spencer rotary microtome, model 820, equipped with a Spencer thin-section adapter so that the unit of advance is approximately 1/20 of the calibrated value. The microtome is set to give the thinnest sections possible. A special copper collar is fitted to the back of the metal microtome specimen holder in such a way that ice water can be kept continually flowing through it, as described by BEAMS *et al.*[2] By conduction the block of tissue is kept at approximately 14° C. A small cardboard container, coated with paraffin, and filled with an ice-chilled mixture of 45% ethyl alcohol and 2% glycerine, is attached to the front of the microtome knife. The ribbons of paraffin and collodion-impregnated tissue float on this mixture during sectioning and do not jam on the microtome knife.

The ribbons are flattened on warm water (45° C), placed on a slide, and dried. The slide is flooded with benzene (to remove the paraffin and collodion), then amyl acetate, immersed in 0.03% collodion in amyl acetate, and dried. The sections adhere to the glass throughout this process and are embedded in a film of collodion. The slide is then dipped in distilled water causing the film to float off on to the surface of the water. Screens are then placed over the portions of the film containing sections and are finally picked up and handled by usual techniques. The screens containing the tissues are exposed to 2% osmic acid vapor for 10 minutes, dried, and shadowed with uranium.

The electron microscope used is an RCA Model E.M.U. equipped with a selfbiased gun and an intermediate lens giving a range of magnification from about 1,000 to about 20,000 diameters.

RESULTS

Light Microscopy

Dark-field microscopy

The first symptom of virus infection of digestive cells of European spruce sawfly larvae observed under the dark-field microscope is a pale-blue refraction of the entire nucleus. The particles causing this refraction are too small to be seen. Polyhedra appear later as small refractive granules. As they increase in size, they become more refractive changing from pale-blue to white. There is no evidence of a ring zone of small refractive granules as described in infections of lepidopterous larvae and observed by the author in studies of a polyhedral virus of the spruce budworm, *Choristoneura fumiferana* (Clem.).

Sectioned and stained tissues

A normal nucleus of a digestive cell of the mid-gut epithelium of a European spruce sawfly larva is shown in Fig. 1. The chromatin, in the form of a network, stains violet by FEULGEN's reaction. A large number of nucleoli, which stain only with the counter stain (light green), are dispersed throughout the nucleus.

The first symptom of virus infection, observed in the study of sections, is a swelling of the nucleus and coagulation of the chromatin to form several masses. The polyhedra appear as minute granules, deeply stained by haematoxylin, within the chromatic masses (Fig. 2). The nucleoli remain unchanged except for a slight swelling.

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Fig. 1–5. Nuclei of mid-gut epithelia of European spruce sawfly larvae. (a) nucleolus; (b) chromatin; (c) immature polyhedra. Fixative, BOUIN's fluid; stain, HEIDENHAIN's iron haematoxylin. × 1800.

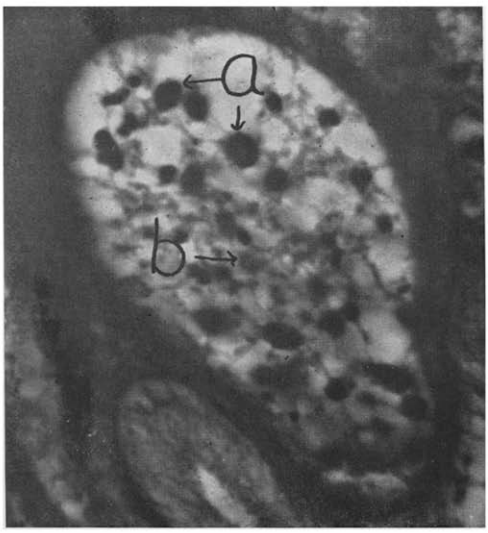

Fig. 1. Normal nucleus

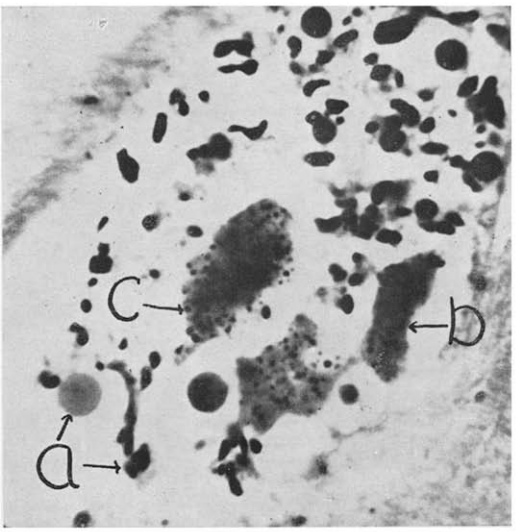

Fig. 2. Virus-infected nucleus showing coagulated masses of chromatin and small deeply-stained immature polyhedra within the chromatic material

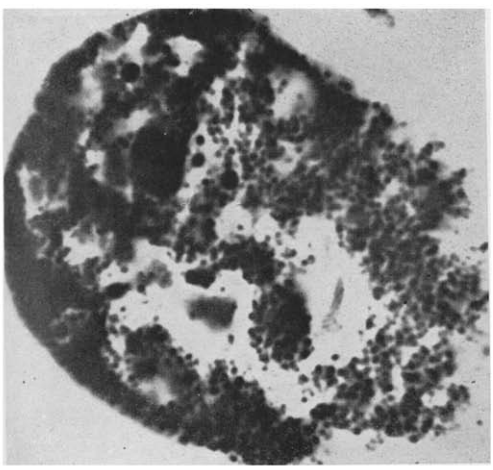

Fig. 3. Virus-infected nucleus filled with deeply-stained immature polyhedra

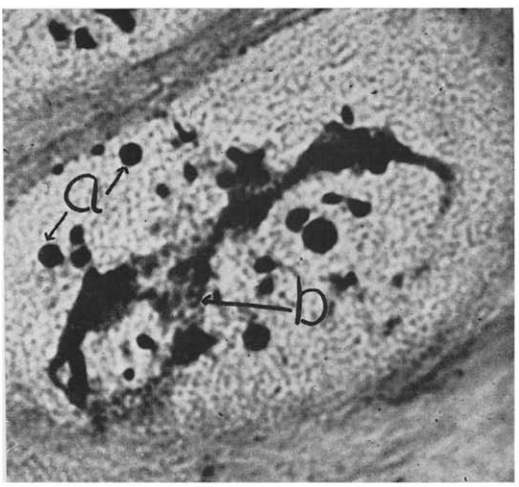

Fig. 4. Virus-infected nucleus with unstained immature polyhedra showing remnants of nuclear material crowded into the center of the nucleus

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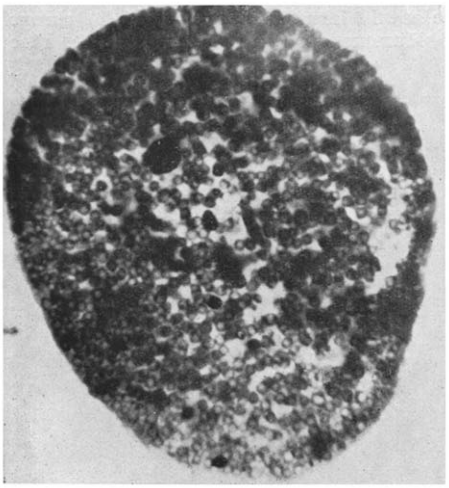

Fig. 5. Virus-infected nucleus showing stain-resistant mature polyhedra

Later the polyhedra increase in number and size and are found throughout the nucleus (Fig. 3). They still stain intensely with iron haematoxylin. It is possible to stain only the chromatin and nucleoli leaving the immature polyhedra unstained. This frequently occurs when the iron haematoxylin stain is insufficiently ripened. The remnants of the nuclear material are thus shown to be crowded towards the center of the nucleus (Fig. 4).

At the end of the infection process, the nucleus is filled with mature polyhedra which are very resistant to haematoxylin stain. The nucleus also contains enlarged nucleoli and remnants of the chromatic material (Fig. 5).

These studies indicate a gradual maturing of the polyhedra and a change in the nature of the polyhedra at maturity which makes them resistant to staining by iron haematoxylin.

Electron Microscopy

Purified polyhedra and virus particles

An electron micrograph of purified polyhedra of the European spruce sawfly is shown in Fig. 6. The average size of the polyhedra is about 1.0 micron. They are typically cuboidal with rounded corners.

Rod-shaped particles similar to the virus particles isolated from the polyhedra of several lepidopterous insects and spherical particles in which the rods are assumed to develop were obtained by dissolving purified polyhedra in weak alkali (Fig. 7). Note encircled spherical particle which contains a rod in a curled position.

The rods average 250 × 50 mμ. The spheres vary in size from less than 80 to more than 160 mμ in diameter.

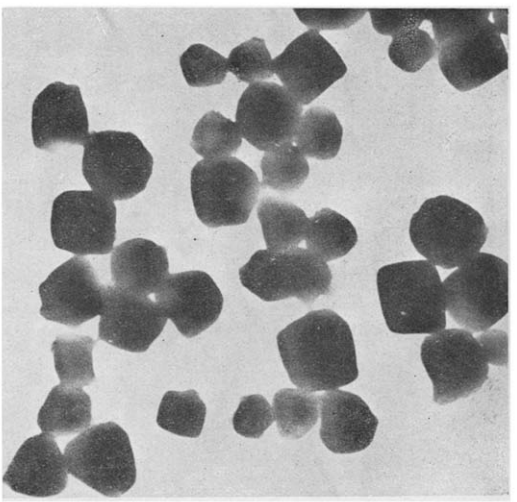

Fig. 6. Electron micrograph of purified polyhedra of the European spruce sawfly. × 12,000.

A study of thin sections

Thin sections were prepared of cells in various stages of infection and examined under the electron microscope. Most of the sections were prepared from cells in an early stage of infection in an attempt to detect virus multiplication before polyhedral formation,

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at the time when the nucleus shows a slight swelling or chromatin coagulation. A cell in an early stage of infection before polyhedral formation is shown in Fig. 8 (12,500 ×) and Fig. 9 (25,000 ×). Particles similar to those isolated from purified polyhedra can be detected (compare encircled spherical particle of the purified virus preparation in Fig. 7 and encircled spheres and rods in Fig. 8 and 9). All nuclei sectioned at this stage of infection have shown a large number of minute spherical particles. Evidences of rods occurring singly and adjacent to empty spherical membranes from which they had apparently escaped were obtained.

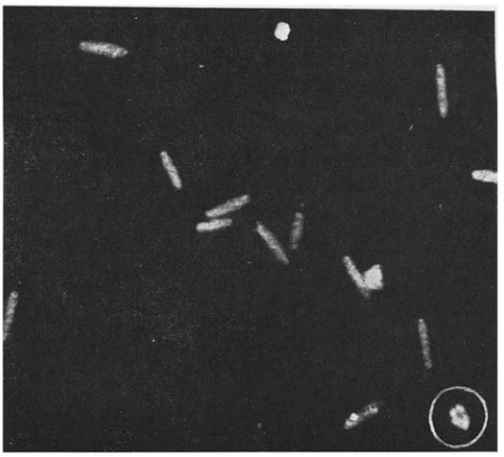

Fig. 7. Particles isolated by dissolving purified polyhedra in weak alkali. Encircled spherical particle contains a rod in a curled position. Positive print. × 37,500.

BERGOLD[7], of this laboratory, isolated infected blood cells of the silkworm, *Bombyx mori* L., ruptured the nuclear membranes, and was able to demonstrate rod-shaped and spherical particles which had escaped from the nuclei.

These data obtained from a study of thin sections of the digestive cells of European spruce sawfly larvae as well as those from isolated blood cells of the silkworm by BERGOLD, indicate that the virus multiplies in the free state within infected nuclei before the formation of polyhedra and that the same developmental stages of the virus occur both in the free state and within polyhedra.

Studies of thin sections further indicate that polyhedra arise as ultra-microscopic bodies and that multiplication and development of the virus particles also takes place within growing polyhedra.

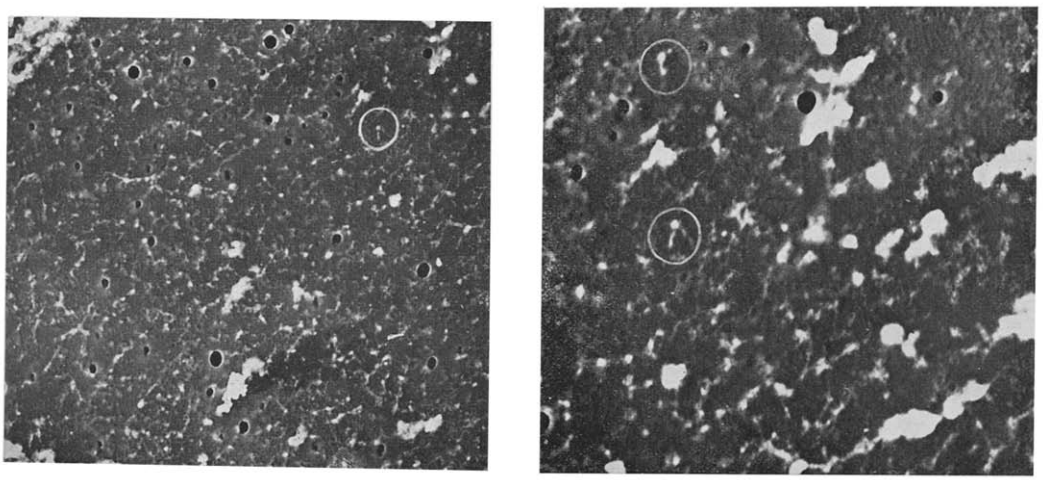

Fig. 8–9. Thin section of virus-infected nucleus before polyhedral formation showing particles similar to those isolated from polyhedra. Encircled particles contain rods in curled positions. Positive prints. Fig. 8. × 12,500; Fig. 9. × 25,000.

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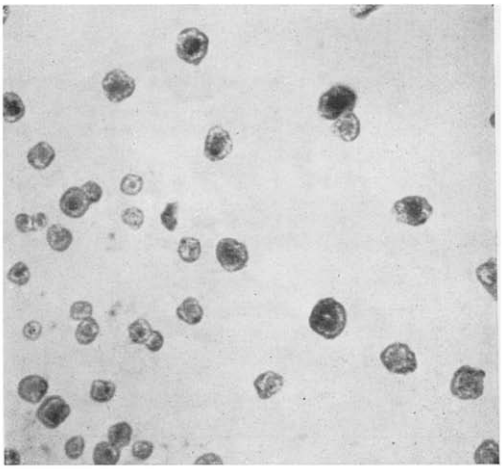

Fig. 10. Immature polyhedra at a very early stage of development extracted from an infected cell. × 25,000.

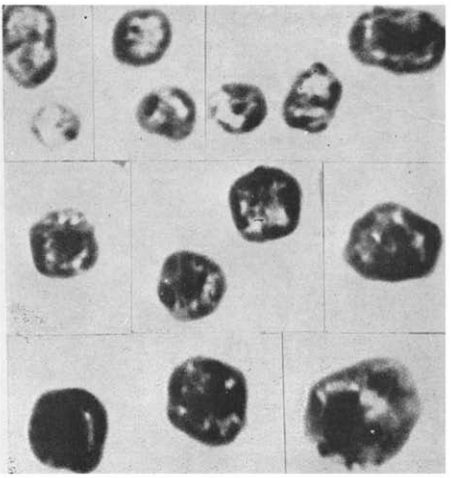

Fig. 11. Selected immature polyhedra in very early stages of development showing spherical particles within the polyhedra and an increase in the number and size of these particles in growing polyhedra. × 75,000.

Material extracted from an infected cell shows the type of body observed in thin sections (Fig. 10). These bodies are regarded as very young polyhedra. They are very similar in shape to the much larger polyhedra obtained from virus-killed larvae (Fig. 6). They contain minute spherical particles which increase in size and number as the polyhedra increase in size (Fig. 11). The smallest polyhedra illustrated measure about 160 mμ in diameter and contain spherical particles about 20 mμ in diameter. These small particles may be elementary particles of the virus.

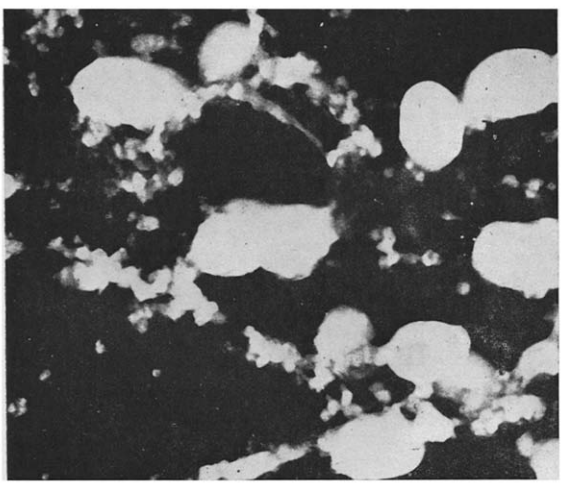

Fig. 12. Thin section of an alkali-treated nucleus showing the disintegration of immature polyhedra. Particles which escaped from partially dissolved polyhedra are chiefly spherical in shape. × 25,000.

It is only at a very early stage of polyhedral development that particles can be observed within the polyhedra which rapidly become opaque. It will be necessary to dissolve polyhedra at various stages of their development after they become opaque to study the type of particles they contain. Preliminary studies of this nature have been made. Entire alimentary tracts of European spruce sawfly larvae, at an early stage of infection, were immersed in 0.01 *M* Na_2CO_3 for several hours and thin sections of the alkali-treated tissues were prepared. Fig. 12 is an electron micrograph of a thin section of a virus-infected nucleus and shows the

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disintegration of swollen immature polyhedra. The particles escaping from the polyhedra are chiefly small spheres and not rod-shaped particles.

Recently SMITH AND WYCKOFF[18] reported that the polyhedra formed in some virus-infected lepidopterous larvae contain only spherical particles. They suggested that the virus diseases of insects which produce crystal-like polyhedral inclusions may fall into at least two groups: those which exhibit rod-shaped particles and those which exhibit spherical particles. The studies on the disease of the European spruce sawfly indicate that a polyhedral disease which exhibits rod-shaped particles at one stage of the infection process may exhibit only spherical particles at an earlier stage.

SUMMARY

Polyhedral bodies, ultimately averaging 1.0 μ in diameter, are formed in the nuclei of the digestive cells of the mid-gut epithelium of virus-infected European spruce sawfly larvae (Order Hymenoptera). Infected nuclei increase in size and the chromatin coagulates to form several masses. The polyhedra appear, under the light microscope, as minute granules within the chromatic masses. Later they appear throughout the nucleus, gradually increase in size, and become resistant to staining.

Rod-shaped virus particles with dimensions of about 250 × 50 mμ and spherical particles varying in size from less than 80 to more than 160 mμ in diameter were isolated from purified polyhedra. Particles similar to these were observed in thin sections of infected nuclei before polyhedral formation indicating that multiplication of the virus takes place in the free state.

An electron microscope study of thin sections of infected nuclei and material extracted from infected cells shows that polyhedra arise as ultramicroscopic bodies about 160 mμ in diameter and contain spherical particles about 20 mμ in diameter which increase in number and size as the polyhedra grow. Apparently multiplication and development of the virus particles take place within growing polyhedra as well as in the free state within an infected nucleus.

RÉSUMÉ

Des polyèdres de diamètre moyen 0.1 μ prennent naissance au sein des noyaux des cellules digestives de l'épithélium de l'intestin moyen de larves de la guêpe du pin européenne (ordre Hymenoptera) infectées par un virus. Des noyaux infectés grossissent et la chromatine coagule et s'accumule à plusieurs endroits. Les polyèdres apparaissent au microscope à lumière comme des granules minuscules au sein des accumulations chromatiques. Plus tard, ils apparaissent partout dans le noyau, grossissent graduellement et deviennent résistants à la coloration.

Nous avons isolé à partir de polyèdres purifiés des bâtonnets dont les dimensions étaient de 250 × 50 mμ environ, et des particules sphériques variant de moins de 80 jusqu'à plus de 160 mμ de diamètre. Des particules semblables ont été observées dans des coupes minces de noyaux infectés avant la formation des polyèdres, ce qui indique que les virus se multiplient à l'état libre.

Une étude à l'aide du microscope électronique de coupes minces de noyaux infectés et de matériel extrait de cellules infectées a montré que les polyèdres prennent naissance comme corps ultra-microscopiques (diamètre: 160 mμ environ) et qu'ils contiennent des corps sphériques (diamètre: 20 mμ environ) qui augmentent en nombre et en taille au fur et à mesure que les polyèdres grossissent. Apparemment, la multiplication et le développement des virus a lieu aussi bien à l'intérieur des polyèdres grossissant que à l'état libre au sein d'un noyau infecté.

ZUSAMMENFASSUNG

Polyeder durschnittlich 1.0 μ im Durchmesser, bilden sich in den Kernen der Verdauungszellen des Mitteldarm — Epithels virusinfizierter Larven der europäischen Fichtenblattwespe. (Order Hymenoptera). Infizierte Kerne nehmen an Grösse zu und das Chromatin koaguliert zu mehreren Ballen. Die Polyeder erscheinen, unter dem Lichtmikroskop, als winzige granula innerhalb der Chromatinballen. Später erscheinen diese überall im Kern, vergrössern sich schrittweise, und werden farbresistenter.

Stäbchenförmige Teilchen mit Dimensionen von etwa 250 × 50 mμ and sphärische Teilchen, im Durchmesser von weniger als 80 bis über 160 mμ variierend, wurden aus gereinigten Polyedern isoliert. Ähnliche Teilchen wurden in Dünnschnitten von infizierten Zellen vor der Polyederbildung beobachtet, was anzeigt, dass die Vermehrung des Virus im freien Zustand stattfindet.

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Eine elektronenmikroskopische Studie an Dünnschnitten von infizierten Kernen und aus infizierten Zellen extrahiertem Material lässt erkennen, dass die Polyeder als ultramikroskopische Körperchen (von etwa 160 mμ Durchmesser) entstehen und sphärische Teilchen (von etwa 20 mμ Durchmesser) enthalten, welche an Zahl und Grösse zunehmen während die Polyeder wachsen. Auscheinend findet Vermehrung und Entwicklung von Virus-Teilchen sowohl innerhalb wachsender Polyeder als auch im freien Zustand innerhalb eines infizierten Kernes statt.

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