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A Granulosis Virus of the Fruit Tortrix, Adoxophyes orana F. v. R. (Lep., Tortricidae)

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A granulosis virus *(Baculovirus* sp., subgroup B), isolated from a moribund larva of *Adoxophyes orana* from an apple orchard in Switzerland has been investigated. The virus develops in the adipose tissue only. Two types of infection can be distinguished: (1) the relatively quickly developing nuclear type, infecting about 60% of the cells, which corresponds to the classical granuloses with development of the virions in the nucleus and, after disruption of the nuclear envelope, in the nuclear field, and (2) the newly described cytoplasmic type, infecting about 40% of the cells, with the relatively slow development of virions and capsules in cytoplasmic enclaves surrounded by a membrane, in cells with a healthy nucleus. The infected larvae die usually in the last larval instar, independent of the time of infection in an early or a late larval instar.

The summer fruit Tortrix, *Adoxophyes orana* F. v. R. [= *A. reticulana* (HB.)] (Lepidoptera, Tortricidae) is one of the most important pests of the apple and pear cultures of the Middle Valais in Switzerland, where 2-3 annual treatments with insecticides (organophosphates or pyrethroids) are necessary to protect the fruit against the attacks of the insect. These chemicals are not specific. Therefore they also attack the natural enemies and favor the development of other pests, such as the psylles *(Cacopsylla pyri)* on pear trees and spider mites on apple and pear trees. Specific biological or biotechnical methods are still to be developed. Concerning microbial control PONSEN & DE JONG (1964) were the first to test a polyhedrosis virus as a control agent of *A. orana.* YAMADA & OHO (1973) and SHIGA *et al.* (1973) multiplied a granulosis virus isolated from the tea-strain of *A. orana* and applied it successfully against the apple-strain in orchards in Japan.

This paper presents the first data on the pathogenicity and the pathogenesis as well as electron microscopic investigations on the cytopathology of a granulosis virus *(Baculovirus,* subgroup B) found in a natural population in the Middle Valais (Switzerland).

MATERIALS AND METHODS

Insects

The larvae of *A. orana* used in our experiments were reared in the laboratory at a temperature of 25 °C and 70% RH on modified semisynthetic medium after DE JONG (1968), i. e. without WESSON's salt mixture and replacing carboxymethylcellulose by cellulose powder. In order to preserve the natural behaviour and

reaction of the larvae of our laboratory strain to insecticides and pathogens, the strain is renewed every year from larvae collected in orchards of the Valais.

Viruses

The granulosis virus (GV) strain used was isolated from a moribund larva of *A. orana* collected at Ardon (Valais). The virus was multiplied in larvae of our laboratory strain. Dead larvae were homogenized and the resulting suspension was filtered trough fine meshed gauze. This primary suspension was kept in the refrigerator and was used as base for all virus dilutions used in our experiments. The virus concentration was determined as capsules/ml by the method of BENZ (1964).

The nuclear polyhedrosis virus (NPV) of *A. orana* was received from Dr. M. PONSEN of Wageningen (Netherlands) and kept in the same refrigerator as the GV suspension.

Infection

Larvae of *A. orana* were usually infected at the beginning of the third instar (10–11 days after eclosion from the eggs) by putting the insects on small pieces of the rearing medium previously dipped in a virus suspension of a given concentration. After 6 days the larvae were removed from the contaminated medium and placed individually in small plastic boxes, containing non-contaminated medium.

Dose-mortality experiments were made with lots of 40–50 third instar larvae (L_3) per GV concentration and experiment. Seven virus concentrations ranging from 2×10^3 to 6.5×10^6 capsules/ml have been tested in two independent experiments (3 concentrations have been repeated). The results were analyzed with a computer program for probit analysis by DAUM & KILLCREAS (1966).

Other infection experiments were made with L_1, L_2 and L_4. They were neither analyzed nor reported in detail here.

Preparations for the electron microscope

Tissues from larvae killed on the 4th and each following day up to the 12th day and on the 19th day after infection were prepared for the electron microscope. Control larvae corresponding to the lots of the 9th to the 12th day and of the 19th day were equally prepared.

The tissues were fixed in paraformaldehyde (KARNOWSKY) for 3–4 h at room temperature, rinsed in 0.1 molar cacodylate buffer at pH 7.0, and fixed again for 1.5 hrs in 1% OsO_4 in MICHAELIS buffer at room temperature. The tissues were then dehydrated with acetone, embedded in expoxy resin (ERL), and cut.

The tissue sections were mounted on copper grids and contrasted with uranylacetate and lead citrate. They were examined in a Philips EM 300 electron microscope.

RESULTS

Dose Mortality

Experiments with the different larval instars showed that the first instar (L_1) was most sensitive and that each subsequent instar was less sensitive to infection with the granulosis virus, the fourth instar larvae being most tolerant. Experiments

were mainly conducted with lots of 40-50 third instar larvae (L_3). Table 1 shows some calculated dose mortality values resulting from infections of L_3 with 7 different concentrations of GV.

Tab. 1: Calculated lethal concentrations (LC = capsules/ML) of the GV of *A. orana* fed to third instar larvae (10 experiments, 7 GV concentrations, 40-50 L_3 per concentration).

LC	Calculated value	95% confidence limits lower	upper
10	$6.2 \cdot 10^2$	$2.4 \cdot 10^2$	$1.6 \cdot 10^3$
30	$5.7 \cdot 10^3$	$3.2 \cdot 10^3$	$1.0 \cdot 10^4$
50	$2.7 \cdot 10^4$	$1.8 \cdot 10^4$	$4.0 \cdot 10^4$
70	$1.2 \cdot 10^5$	$8.0 \cdot 10^4$	$1.9 \cdot 10^5$
90	$1.2 \cdot 10^6$	$5.5 \cdot 10^5$	$2.4 \cdot 10^6$

Incubation time

The incubation time of the granulosis is extremely long. The first symptom, i. e. larvae turning slightly opaque, appears not before 10-15 days after infection. Infected larvae are still feeding when all the controls have pupated (Table 2) and the first moths eclose. But once the first larvae die the disease will progress rather rapidly in the infected group. The incubation time seems to be dose-independent, since the medium time to the death of the dying larvae in 7 experiments with different virus doses (30 L_3 per experiment) varied only from 23.5 to 25.6 days. The survival values given in Table 2 are the average values of an experiment with 200 larvae infected with a dose of GV leading to more than 90% mortality.

Tab. 2: Calculated survival time (ST) of 210 infected larvae and approximative time to pupation of 500 untreated control insects.

ST	infected larvae	Pupation of control	
70%	23.5 ± 1.0 d	30%	13 d
50%	25.0 ± 1.5 d	50%	14.5 d
30%	26.5 ± 1.7 d	70%	16 d
10%	29.0 ± 1.6 d	90%	18 d

Although the young larvae are more susceptible than the old ones, the incubation time is longer for young larvae, since all infected larvae live at least up to the fourth and most of them to the fifth instar. Larvae infected after hatching (L_1) die on the average 35 days after infection, those infected as 5-6-day-old L_2 die 30 days after infection, and 10-day-old larvae 25 days after infection. However,

during the last days of their lives the larvae hardly move or respond to external stimuli.

Pathogenesis in the insect

The observations in the electron microscope allow to follow the development of the granulosis in the body of the insect. The virus multiplies in the adipose tissues, as can be seen in the tissue preparations up to the 19th day after infection; the epidermis, the tracheal matrix and the muscles on the other hand are not affected. No pathologic signs can be seen during the first 6 days after infection. On the seventh day the first nucleocapsids appear within the infected nuclei (Fig. 1) and on the 9th day many of the nuclear envelopes disrupt. Capsules and virus particles at different stages of development appear in the cytoplasm and surround the nuclear area where the nucleocapsids are synthesized (Fig. 2).

Besides these cells, which are typical for most granuloses known up to the present day, another type of infected cells can be observed. Their nuclei appear to be normal and are surrounded by intact nuclear envelopes, but the cytoplasm contains clusters of capsules (Figs. 3–4). If the development of this phenomenon is followed up right from the beginning, well contrasted vesicles with diameters of 0.4–0.6 microns can be distinguished first (Fig. 5). These vesicles are surrounded by a membrane and also occur in healthy tissues (Fig. 5a); they may be proteinaceous inclusions. On the tenth day the first modifications can be observed within the vesicles (Figs. 5b–c), which begin to swell. On day 12 the first naked (Figs. 5d–f) and encapsulated nucleocapsids appear in the vesicles (Figs. 5g–i). On day 19 all the developmental stages up to clusters of mature capsules can be found, still enclosed within the membrane of the enclave in which they formed.

At a later stage of infection, large masses of capsules are found in the completely disordered cytoplasm of cells whose nuclei are still present.

It is also possible to find cells with the characteristics of both types of granuloses, i. e. clusters of capsules in the cytoplasm of cells whose nuclei enclose rod shaped virus particles. Several countings at 19 days after infection showed that about 60% of the cells of the fat body are infected with the nuclear type of the granulosis and 40% with the cytoplasmatic type.

Dimensions of the virus particles and the capsular inclusion bodies

The nucleiocapsids were measured in ultrathin sections. For the measurements only particles longer than 150 nm were selected, i. e. those which had been cut lengthways. The nucleocapsids are 200–230 nm long and have a diameter of 34–36 nm. The capsules measure about 390 by 180–200 nm.

Abnormal inclusion bodies

Among the ovocylindric capsules produced in the course of the infection, elongated forms, composed of 2 or more units and containing more than one particle can be seen. The capsular protein also forms abnormal aggregates, scattered all over the cytoplasm, which are generally not associated with virions. These abnormal or aberrant forms can make up to 10% of the inclusion bodies recorded on the section of an infected cell. In certain cells the production of the virions appears normal, the inclusion bodies are cubic bodies of different size, which may or may not contain a virus particle.

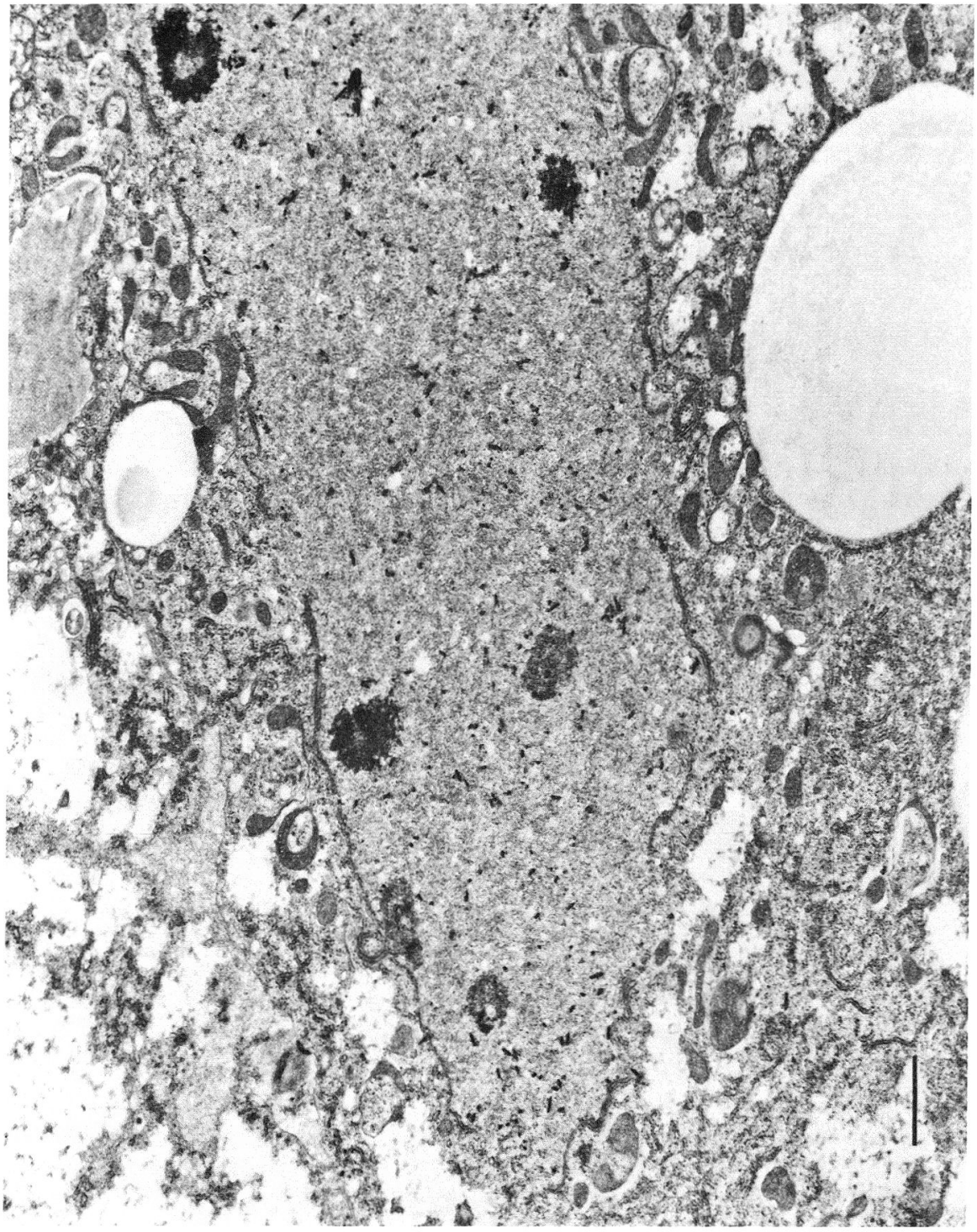

Fig. 1: Nuclear type of granulosis of *Adoxophyes orana*. Multiplication of virus in the nucleus of a fat body cell. The nuclear envelopes are ruptured at several places and the virions begin to migrate into the cytoplasm. Bar = 1 micron.

Combination of NPV and GV

In all our experiments with the NPV the dose of the virus used was too high, leading to almost 100% mortality. When to the NPV doses of the GV were added, which alone would give 50–90% mortality, the mortality remained near 100%, but the incubation period was prolonged by 10–12 days.

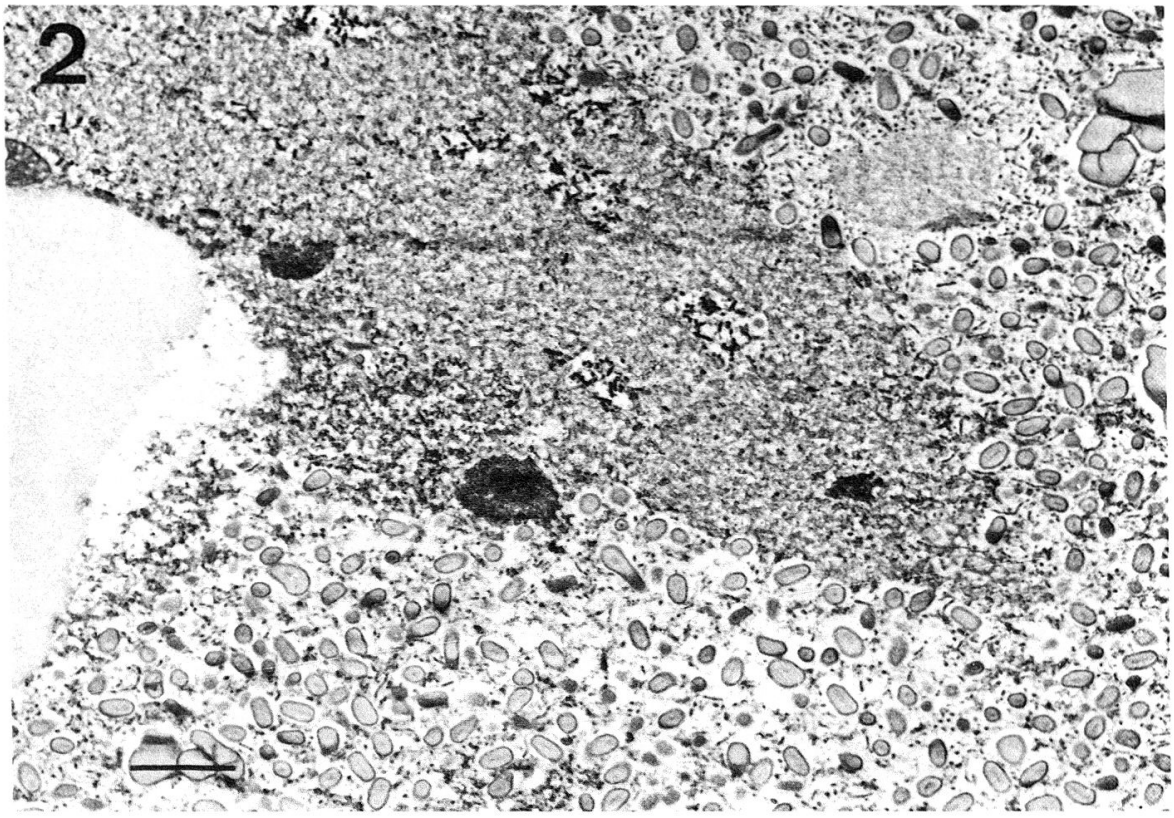

Fig. 2: More advanced stage of nuclear type of granulosis. The nuclear envelopes have fully disappeared and the disorganized cytoplasm contains a large number of capsules. Bar = 1 micron.

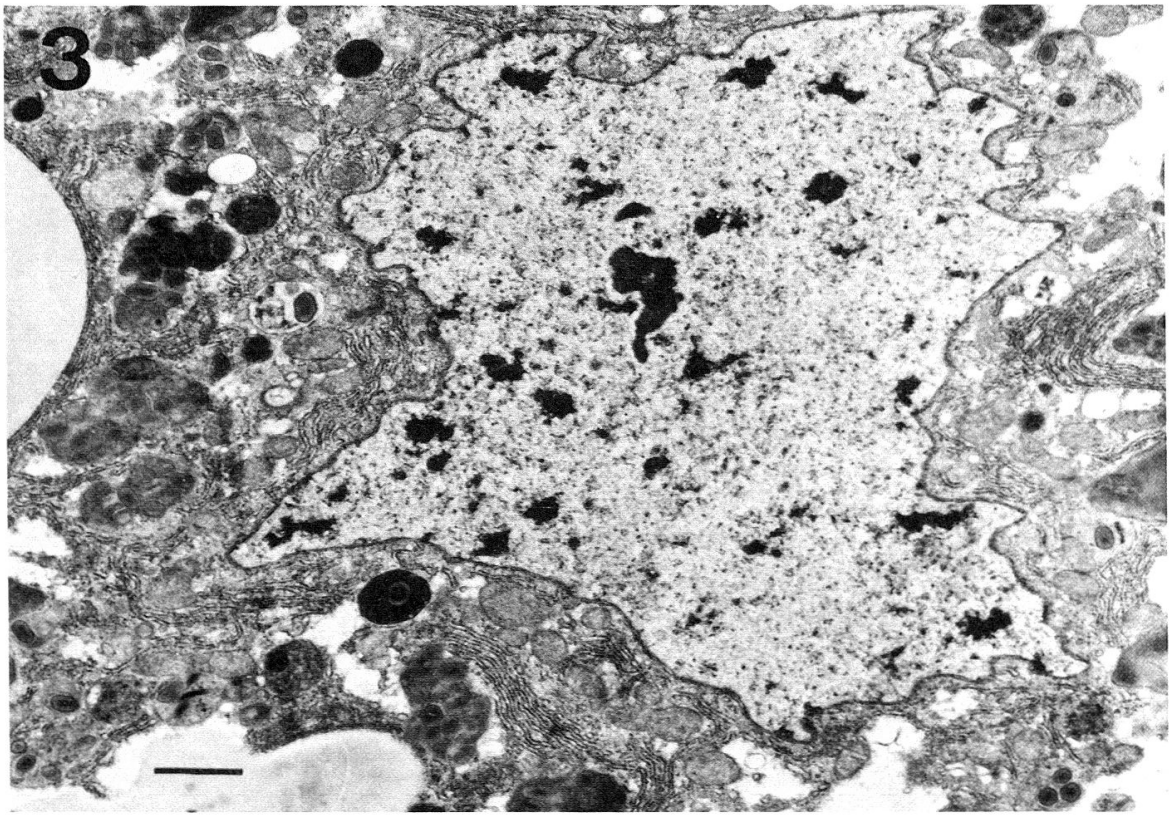

Fig. 3: Cytoplasmic type of granulosis of *A. orana*. Multiplication of virus in cytoplasm of fat body cell. The cytoplasm surrounding the intact nucleus contains deeply stained vesicles which enclose a variable number of viruses. Bar = 1 micron.

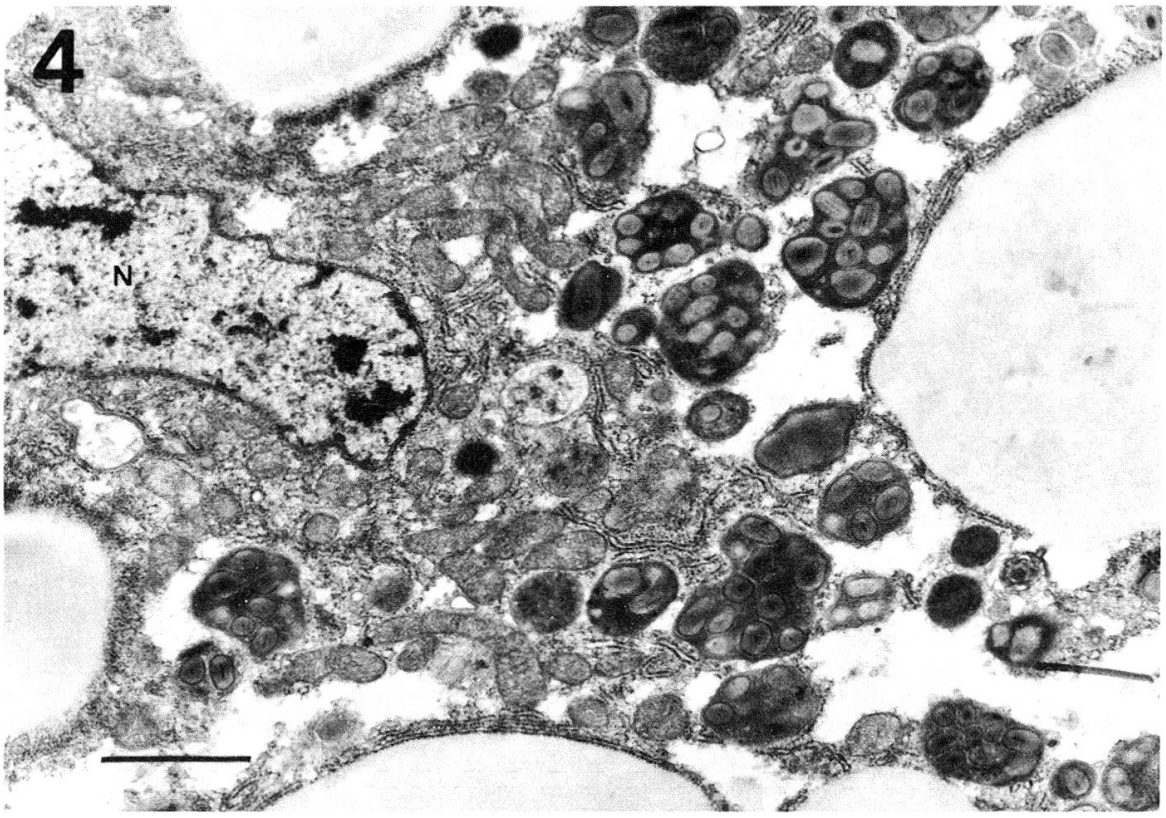

Fig. 4: Different developmental stages of granulosis virus within proteinaceous enclaves (vesicles) of the cytoplasm. N = nucleus. Bar = 1 micron.

DISCUSSION

The most striking features of this granulosis virus of *A. orana* are the very long incubation time, the restriction of the disease to the fat body, and the strictly cytoplasmic development of the virus in 40% of the infected cells. Whatever the dose of the virus and the age of the larvae at the time of infection are, larvae generally die in the last instar only. The diseased larvae are obviously not able to pupate. Thus the Swiss granulosis looks very much the same as the Japanese granulosis described and studied by JAMADA & OHO (1973), SHIGA *et al.* (1973), and OHO (1974). The dimensions of the capsules reported by the Japanese workers are also the same as those found in the Swiss isolate, although there seems to be a difference concerning the dimensions of the nucleocapsides. Whereas the Japanese workers reported the mean dimension of 290/72 nm for their isolate, we found 200–230/34–36 nm in our isolate. However, a comparison of ultrathin sections of capsule samples of the two isolates by C. FLÜCKIGER (unpublished) showed no difference in the morphology and the dimensions of the two virus isolates.

The abnormal forms of the inclusion bodies found in *A. orana* have also been described for other granuloses (ARNOTT & SMITH, 1968; MAIGNAN, 1975). According to the last mentioned author abnormal aggregates of capsule protein in granulosis infected larvae of the codling moth are characteristic for larvae which will die late in the last instar. The presence of abnormal inclusion bodies is therefore a consequence rather than the cause of the slow development of the granulosis in *A. orana.*

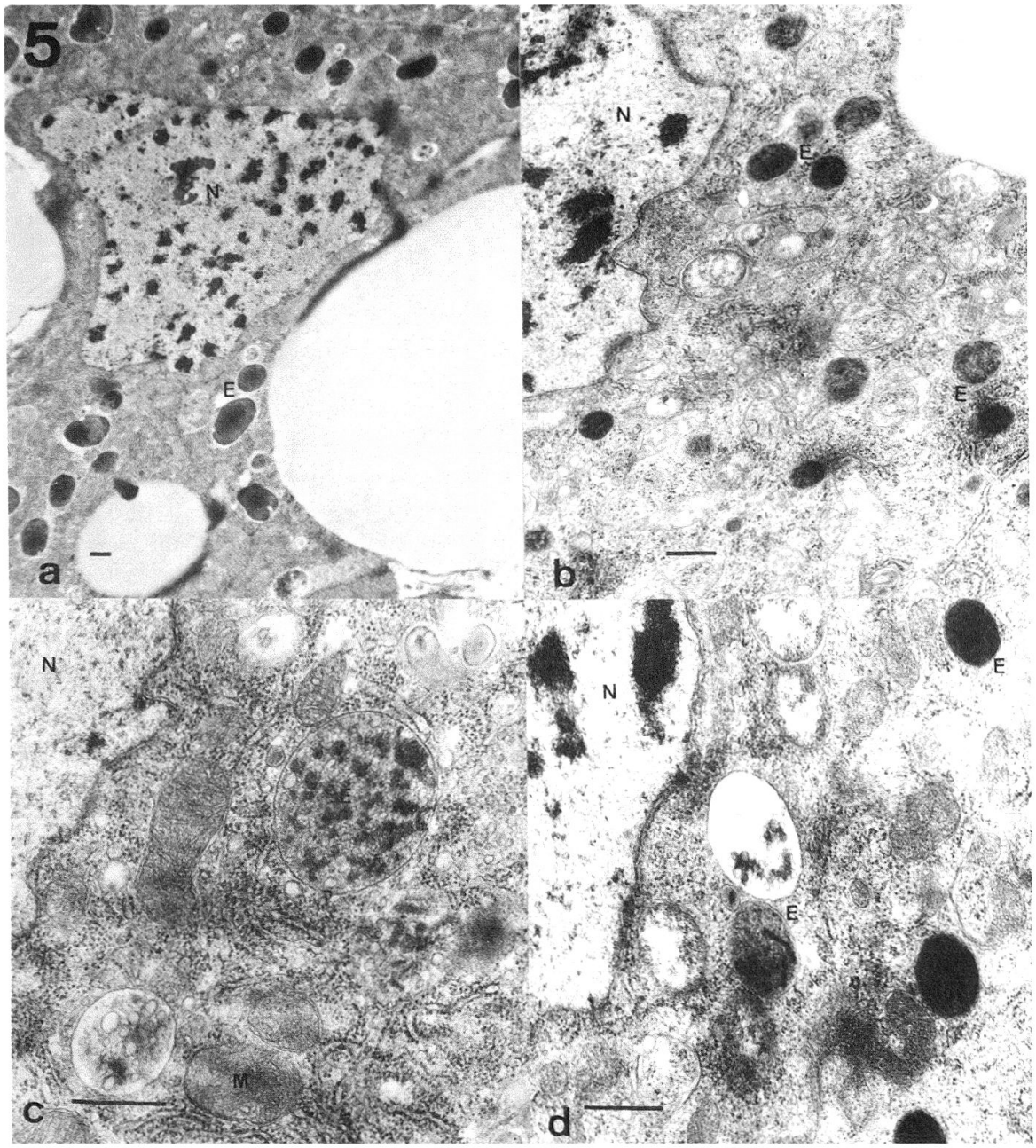

Fig. 5: Cytopathologic changes taking place during virus development in the proteinaceous enclaves of the cytoplasm. (a) healthy cell with its nucleus, surrounded by deeply contrasted enclaves; (b) first modifications in enclaves of cytoplasm of infected cell; (c) detail of infected enclave; (d) first virions appear in the enclaves. N = nucleus, E = proteinaceous enclave, M = mitochondrium. Bars = 1 micron.

In infected fat body cells of *A. orana* the development of the virus seems to follow two different ways. The first mode is the classic development of virions within the nucleus, leading to the rapid destruction of the nuclear envelopes, followed by an enormous multiplication of the virions within the nuclear field and the encapsulation of the virions within the cytoplasm, as described by SMITH & RIVERS (1956), HUGER & KRIEG (1961), WATANABE & KOBAYASHI (1970), BENZ & WÄGER (1971), WÄGER & BENZ (1971), and MAIGNAN (1975). We call it the nuclear type of granulosis. The second mode described here leaves the nucleus intact, follows a slower rhythm, and the development of the virions and their

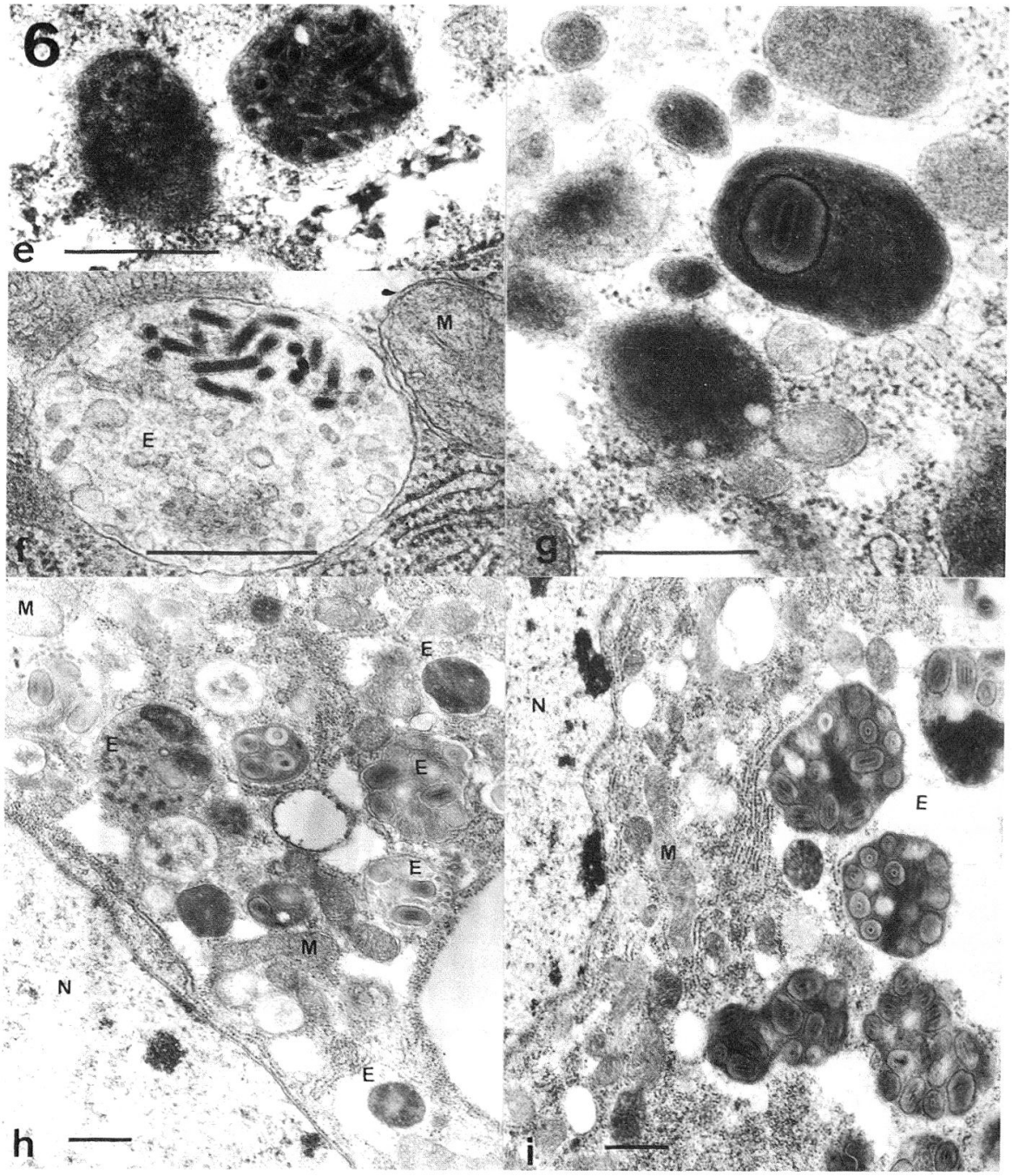

Fig. 6: Same as Fig. 5. (e-f) details of enclaves containing virions; (g-i) more advanced stages of the enclaves enclosing an increasing number of encapsulated viruses. Bars = 1 micron.

encapsulation takes place exclusively in the cytoplasm. Although SMITH (1976) writes that the granuloses develop in the nucleus or in the cytoplasm and cites HUGER (1961) as having shown that the capsules develop at the same time in the nucleus and in the cytoplasm of *Choristoneura murinana,* it is likely that he was referring to the nuclear type of granulosis mentioned above. We therefore think that this paper reports on a new type of granulosis which we call the cytoplasmic type. It is characterized by the formation of nucleocapsides within proteinaceous islets or enclaves of the cytoplasm. Whether or not DNA replication takes place in these enclaves has not been demonstrated yet.

Mixed infections of the nuclear and the cytoplasmic type of granulosis are possible up to a certain point. If the nuclear type is installed first, the cytoplasmic type cannot develop, because the cytoplasm disintegrates too early. In order to develop the cytoplasmic granulosis the virus must either infect alone or sufficiently precede the nuclear type.

From the practical point of view this granulosis cannot be used for pest control by applying it in the conventional sense, i. e. to reduce the damage producing generation. Since the life span and feeding period of the infected larvae is considerably prolonged, they cause more damage than the healthy insects. In this connection it is interesting to note that the long feeding infected larvae do not grow larger and heavier than the healthy. The same has been observed by WHITLOCK (1974) in *Heliothis armigera.* However, the weight of the moribund larvae may depend on the dose used for infection. According to CAMPONOVO (1980) small doses of GV lead to abnormally heavy and high doses to abnormally light last instar larvae of the codling moth, *Cydia pomonella.*

The GV of *A. orana* might be used in an integrated control programme where the effect sought is not the control of the damage producing generation but a reduction of the population density in the subsequent generations. ITO *et al.* (1977) have demonstrated that in Japan one treatment of the first generation has still a good effect in the second generation. Thus the GV of *A. orana* should probably be used with the same strategy as growth regulators are used, i. e. treatment of the overwintering larvae in spring, in order to reduce the larval population below the tolerance level (SCHMID *et al.,* 1978).

From our experiments in which the GV was combined with the NPV no definite conclusions may be drawn yet. However, field experiments by BENZ, FLÜCKIGER and HAAS (unpubl.) with the same combination of viruses also showed a prolongation of the survival time. It seems therefore safe to conclude that the granulosis antagonizes the development of the polyhedrosis to a certain degree. Such an antagonism has been observed earlier by BIRD (1959) and WHITLOCK (1977) in other insect species. Concerning *A. orana* more experiments with different doses of the two viruses should be made.

RÉSUMÉ

Une granulose de capua Adoxophyes orana F. v. R. – Dans ce travail, nous présentons les premiers essais de laboratoire avec une granulose trouvée sur une population naturelle de capua *(Adoxophyes orana = A. reticulana)* du Valais central. Nous étudions, à l'aide de microscope électronique, l'évolution de la maladie dans les insectes infectés et nous essayons de combiner cet agent pathogène avec la polyédrose susmentionnée. Les caractéristiques les plus frappantes de cette granulose de capua sont une virulence assez forte et un temps d'incubation très long. Quelle que soit la dose et l'âge des larves au moment de l'infection, elles meurent en général au dernier stade larvaire. L'infection semble suivre deux cheminements différents. Le premier (ca. 60% des cellules) provoque l'éclatement du noyau et assure une forte multiplication du virus: on peut parler de granulose nucléaire. Dans le deuxième cas (ca. 40% des cellules) l'infection suit un rythme plus lent, ne touche pas le noyau et reste limitée au cytoplasme.

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