Characterization of nuclear polyhedrosis viruses obtained from *Adoxophyes orana* and from *Barathra brassicae*

CENTRALE LANDBOUWCATALOGUS
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Promotor: dr.ir. J.P.H. van der Want, hoogleraar in de virologie
Co-referent: dr.ir. D. Peters, wetenschappelijk hoofdmedewerker

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Mária Jurkovíčová

CHARACTERIZATION OF NUCLEAR POLYHEDROSIS VIRUSES OBTAINED FROM *ADOXOPHYES ORANA* AND FROM *BARATHRA BRASSICAE*

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
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STELLINGEN

I

De kennis over insectevirussen is thans nog zo beperkt, dat aan het op grote schaal toepassen van deze virussen als insecticiden grotere risico's verbonden zijn dan aan het gebruik van chemische middelen.

II

De basis op grond waarvan identificatie van afzonderlijke kernpolyederviruscomponenten kan plaatsvinden, zal gelegd worden door studies naar de herkomst en aard van hun eiwitten.

Beek, C.P. van der (persoonlijke mededeling).
Summers, M.D. en Smith, G.E. (1978). Virology 84, 390-402.
Dit proefschrift.

III

De grote verschillen tussen de door de onderzoekers gekozen experimentele omstandigheden vormen een groter probleem bij de vergelijkbaarheid van de karakterisering van kernpolyedervirussen dan de activiteit van geassocieerde proteolytische enzymen.

Summers, M.D. en Smith, G.E. (1975). J. Virol. 16, 1108-1116.
Eppstein, D.A. en Thoma, J.A. (1975). Biochem. Biophys. Res. Comm. 62, 478-484.
Dit proefschrift.

IV

Zoals het zich thans laat aanzien zal de inmiddels "klassieke" chemische bestrijding een centrale plaats blijven innemen in de geïntegreerde bestrijding van ziekten en plagen.

Kerk, G.J.M. van der (1978). Voordracht symposium "Aspecten van de Agrofarmacochemie", Wageningen, 23 maart 1978.

V

De vorm van symbiontisch levende, stikstofbindende actinomyceten wordt voor een belangrijk deel bepaald door de waardplant en kan daarom niet als taxonomisch kenmerk worden gebruikt.

Becking, J.H. (1970). In "Bergey's manual of determinative bacteriology". (Buchanan en Gibbons, coed.). 701-706.

BIBLIOTHEEK
DER
LANDBOUWHOGESCHOOL
WAGENINGEN

VI

Het aantal gevallen van succesvolle biologische bestrijding van schimmel- en bacterieziekten door inoculatie van plantgoed met aan de pathogenen verwante organismen neemt toe. Het is wenselijk het mechanisme dat hierbij een rol speelt met een meer adequate term aan te duiden dan het algemeen gebruikte "cross-protection".

Melouk, H.A. en Horner, C.E. (1975). Phytopathology 65, 767-769.
Wong, P.T.W. (1975). Soil Biol. Biochem. 7, 189-194.

VII

Vanwege de noodzaak tot bescherming van het grondwater als een van de belangrijkste bronnen voor drinkwatervoorziening dient meer aandacht te worden geschonken aan de factoren die de mobiliteit in de grond reguleren van voor mens en dier pathogene virussen en micro-organismen.

Kool, H.J. (1979). In "Handboek voor Milieubeheer". (Van den Berg e.a., eds.), Band 1 (4), 1-11.

VIII

Noch met de voor handen zijnde resultaten van laboratoriumonderzoek noch met die van karteringsstudies kan bewezen worden dat korstmossen een grotere gevoeligheid dan hogere planten hebben voor en specifiek reageren op afzonderlijke luchtverontreinigende stoffen. Daarom mist de bewering dat korstmossen geschikt zijn als biologische meetinstrumenten van zwaveldioxide iedere grond.

Wit, T. de (1976). Proefschrift, Rijksuniversiteit Utrecht.
Messelink-Beltman, H.A. (1977). In "Korstmossen in Drenthe" (Provinciale Waterstaat van Drenthe, uitgever).

IX

Isolatie van woningen en gebouwen gepaard gaande met een vermindering van de ventilatie, kan zulk een stralingsdosis tot gevolg hebben, dat, volgens de voor de toepassing van kernenergie gebruikelijke risicoschattingen, het aantal longkankergevallen aanzienlijk zal stijgen.

Auxier, J.A. (1976). Health Physics 31, 119-125.
Sources and effects of ionizing radiation (1977), UNSCEAR-rapport.

X

Het zoeken naar een schaap met vijf poten onder sollicitanten naar een bepaalde betrekking, doet vermoeden, dat de eiser zulks doet om binnen de kudde het gemiddelde aantal poten per schaap weer op vier te brengen.

XI

De mens van nu is tegenover hen die later zullen leven verplicht alles te doen, waardoor het leven verrijkt, vereenvoudigd en beter leefbaar wordt.

XII

Ars moriendi, een afscheidsfeest, waarbij de stervende omringd en gesteund door de zijnen, zijn leven voltooide en stierf zoals hij geleefd had, heeft in onze moderne samenleving plaats moeten maken voor de "verzorgde" maar eenzame en absurde klinische dood. Dit betekent niet alleen een verarming voor de stervende, maar ook het ontnemen van een levenservaring voor hen die nog verder leven.

Mária Jurkovíčová

Characterization of nuclear polyhedrosis viruses obtained from *Adoxophyes orana* and from *Barathra brassicae*

Wageningen, 24 oktober 1979.

"Haalve nachten, haile doagen
achter vlinders aan te joachen
da's mooi waark!" zegt Prikkebain,
"'k Mag mie ja in huus nait zain."

Wonderlieke Raaize van
Meneer Prikkebain
deur J.A. Fijn van Draat
(Becht - Amsterdam)

Na pamiatku mojim rodičom
Aan mijn ouders

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In conclusion, I acknowledge my debt to all who in any other way contributed to the final results of this study.

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INTRODUCTION

For many years chemical insecticides have been widely used for the control of insect pests in agriculture, veterinary and public health (Smith, 1975). In nearly 300 insect species resistance to certain types of insecticides have been observed. The resistance seems to increase with the higher and more frequent rates of application. Also levels of more persistent insecticidal residues have increased. At present, resistance in some species has developed to such an extent that chemical insecticides do not give a safe control and therefore they are no longer useful.

There is an urgent need to develop specific and safe pest control methods. Insect viruses, especially baculoviruses, in which the nuclear polyhedrosis viruses (NPVs) and granulosis viruses (GVs) have been grouped together, have many characteristics that ideally suit them for use in integrated control programmes. They are specific and often highly virulent to their hosts (Summers et al., 1975). At this time there are five NPVs in use or near registration for insect control with the authorities in the U.S.A. and Canada. Most of them have a narrow host range, some, however, have a broad range, but the pathogenicity of all is restricted to the class Insecta. Caution is required as the whole host range of most NPVs is not known. It can be expected that the number of known hosts will increase (Vail and Jay, 1973; Stairs and Linn, 1974).

A striking property of the NPVs is the large protein crystal in which the enveloped viruses are occluded (Fig. 1). According to the way viruses are embedded in the polyhedra two groups are distinguished. One group contains many singly embedded virus particles and the other contains bundles of virus particles (Wildy, 1971).

The polyhedra can range in size up to 15 μm and the shape of the polyhedra is controlled according to some authors by the virus (Gershenson, 1959; 1960; Stairs et al., 1966; Arnott and Smith, 1968). However, other authors hold the view that the shape of polyhedra is not controlled by the virus genome (Aizawa, 1961; Bergold, 1947).

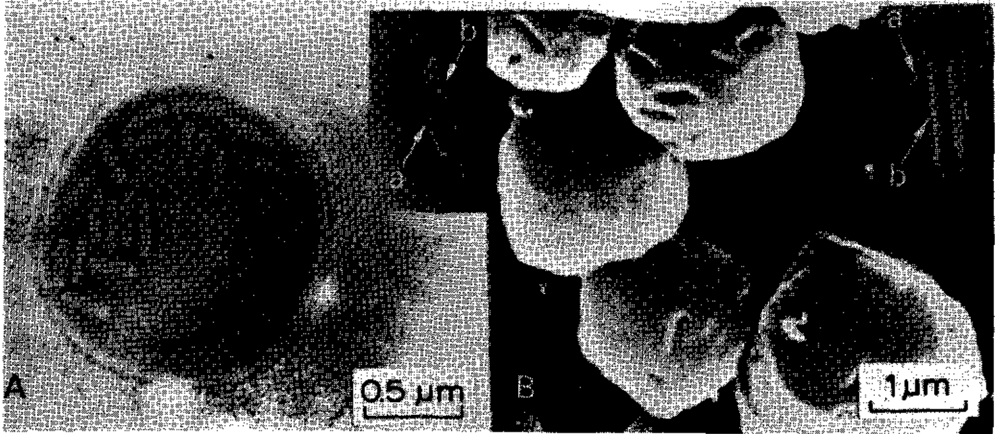

Fig. 1. A partially degraded polyhedron of the *Adoxophyes orana* NPV. Upper right: detail of a single embedded virus particle (A). An undegraded polyhedron of the *Barathra brassicae* NPV. Upper right: detail of a multiple embedded virus particle (B).

The polyhedra can be easily dissolved in a sodium carbonate solution. The polyhedral membrane ruptures, and the virus particles and polyhedral proteins are released.

The structure of the polyhedral membrane has been studied by Summers and Arnott (1969) who showed that the definite presence of a surface structure, unique in morphology, is related to the polyhedral protein. The membrane is characterized by the presence of holes apparently distributed in an at random fashion. This indicates that the membrane structure differs basically from the organization of the polyhedral protein in the polyhedron. The significance of this observation is that the membrane can be considered as a distinct entity at and around the polyhedron. The origin of this membrane is not clear. It has been suggested that it consists of polyhedral protein hardened at the surface of the polyhedron (Wyatt, 1950). Broome (personal communication) has evidence that this membrane consists of polysaccharides.

The virus particles are surrounded by two membranes, designated as outer and inner or intimate membrane (Bergold, 1953a). The virus particles represent about five per cent of the polyhedra. The remaining 95 per cent of polyhedra

consists of protein (Bergold, 1953b). This polyhedral protein, which surrounds the enveloped virus particles and is sometimes designated as polyhedrin (Summers and Egawa, 1973), has an estimated molecular weight of 25,000 to 30,000. It is known, however, that there is an alkaline protease associated with the polyhedra (Yamafuji et al., 1958; 1959) that can complicate the characterization of the polyhedral proteins (Eppstein and Thoma, 1975; Eppstein et al., 1975; McCarthy and Liu, 1976; Payne and Kalmakoff, 1978; Summers and Egawa, 1973; Summers and Smith, 1975).

The genetic material enclosed within the virus particles is a circular, double-stranded deoxyribonucleic acid (DNA) molecule, in the form of a superhelix (Summers and Anderson, 1973; Bud and Kelly, 1977; Burgess, 1977; Harrap et al., 1977; Kelly, 1977; Rohrmann and Beaudreau, 1977). The DNA of these viruses has been further analysed by sedimentation techniques, electron microscopy and reassociation kinetics. These molecules have estimated molecular weights ranging from 50×10^6 to 100×10^6 Daltons. Characteristic fragment patterns are obtained by restriction endonuclease analysis of the DNA of some baculoviruses. The size of the genomes of these baculoviruses was also determined from the molecular weight of these fragments. These analyses were also used to discriminate between baculoviruses. Rohrmann et al. (1978) showed in this way that two viruses pathogenic for *Orgyia pseudotsugata* were different.

Lepidoptera larvae normally become infected with NPV by ingestion of contaminated food. The process of infection involves the dissolution of the polyhedra supposedly by the help of alkaline conditions in the gut. The liberated virus passes through the peritrophic membrane. Two routes may now be followed to infect the different tissues of the larvae. The virus may enter a midgut epithelial cell and migrate to the nucleus where replication takes place. The infection can then spread to other tissues by newly formed particles (Harrap and Robertson, 1968; Harrap, 1970). It may also be possible that the virus does not enter the epithelial cells but pass through spaces between these cells (Tanada and Leutenegger, 1970; Granados, 1978).

Probably, the most efficient method to disperse the virus in nature is by flight or migration of virus-infected insects (Tanada et al., 1964). When the virus infection is introduced into an insect colony, virus may spread horizontally as well as vertically. Generation-to-generation spread of NPVs occurs through the eggs. This may occur by at least two different ways. In the first way, viz. transovum transmission, the virus occurs at the surface of the egg, presumably in the form of polyhedra. Contamination may occur in the female genitalia (Martignoni and Milstead, (1962).

Transovarial transmission, the second way, is explained by the presence of virus in the egg. This type of transmission was first suggested for *Bombyx mori* (Conte, 1907). The occurrence of transovarial transmission of insect viruses is generally not accepted (Smith, 1967). However, the sudden outbreaks of polyhedrosis or granulosis in seemingly healthy laboratory insect populations, and the persistent infection of an insect cell line suggests the presence of endogenous viruses in insects and cells (Smith, 1967, Granados et al., 1978). This suggestion is supported by the observation that surface sterilization of eggs did not always lead to healthy colonies (Vago et al., 1961a and b; Harpaz and Ben Shaked, 1964). On the other hand eradication of NPV by surface sterilization of *B. brassicae* eggs has been reported by Gröner (1976). The state in which the virus is transmitted within the egg and the mechanism involved is unknown at present. The virus DNA may be integrated in the host genome (Yamafuji, 1966; Yamafuji and Hashinaga, 1966) as it is in certain bacteria and in a number of vertebrates (Hayes, 1969; Fenner et al., 1974). It may also be possible that virus or even polyhedra are introduced into the egg by seminal fluids after copulation. The larvae will then consume the virus with the content of eggs, and when the initial dose is small the virus can persist in the progeny as a latent infection.

Several authors have reported the occurrence of latent virus infections in healthy populations. These infections have been activated by several methods. For example, the application of high or low temperatures caused the death of larvae by polyhedrosis (Kurisu, 1955; Aruga, 1957; Steinhaus, 1960; Aruga and Watanabe, 1961; Tanaka and Aruga, 1963). However, there are some doubts about these results because Kurata (1967) pointed out that the experiments were carried out under insufficiently aseptic conditions. The effect of some chemicals has also been investigated (Gershenson et al., 1959; Tanada, 1959; Aruga and Hukuhara, 1960; Steinhaus and Dineen, 1960; Karpov, 1964). The effect of irradiation seems rather uncertain (Aruga and Yoshitake, 1960; Karpov, 1959; 1960). Various authors have reported the induction of polyhedrosis in insects by changing food (Ripper, 1915; Vago, 1951; 1955; David and Gardiner, 1965). Activation of latent infections occurs also by inoculation of larvae with foreign polyhedra (Smith and Rivers, 1956; Smith, 1963; 1964). Here again some doubt exists about the conditions under which the experiments were done.

Hence in the past much work has been carried out to prove that insects may carry latent virus infections but experiments to detect the virus in those insects which presumably carried such an infection have not been performed.

Ponsen and De Jong (1964) observed that the NPV of *B. brassicae* was pathogenic for *A. orana*. This observation was of economic importance because of the size of *B. brassicae* large amounts of *B. brassicae* NPV could be produced and used for the control of *A. orana* in the field. However, they also observed that when *B. brassicae* polyhedra, which contain multiply embedded virus particles, were applied to *A. orana*, the polyhedra produced in *A. orana* contained singly embedded virus particles, which are characteristic for *A. orana*'s own polyhedra. Ponsen and De Jong gave no answer to this riddle.

This observation can be explained in two different ways. When we assume that *B. brassicae* NPV is specific for *B. brassicae* it is possible that the polyhedra of this virus activate a latent infection in *A. orana*. This might then result in the development of a polyhedrosis and the formation of polyhedra with singly embedded virus. It is also possible that *B. brassicae* NPV can infect *A. orana* larvae but that the embedding of virus within polyhedra is controlled by the infected host. This might mean that *B. brassicae* NPV could be used to control *A. orana* in the field.

The aim of the present study was to characterize the NPVs of *A. orana* and of *B. brassicae*, and to analyse whether the NPV of *A. orana* and of *B. brassicae* are either infective in the reciprocal host or activate a latent infection. This study was completed by attempts to demonstrate the presence of viral DNA sequences in DNA extracted from uninfected hosts.

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I PROPERTIES OF THE *ADOXOPHYES ORANA* AND OF THE *BARATHRA BRASSICAE* NUCLEAR POLYHEDROSIS VIRUS AND THEIR POLYHEDRA

SUMMARY

Polyhedra of nuclear polyhedrosis virus (NPV) of *Adoxophyes orana* and of *Barathra brassicae* formed a heterogeneous population in size and shape. The polyhedra of *A. orana* have a globular shape and those of *B. brassicae* are hexagonal or pentagonal in outline. The polyhedra of *A. orana* ranged in diameter from 1 - 2 μm and those of *B. brassicae* 1.5 - 4 μm. After electrophoresis of with sodium dodecyl sulphate treated polyhedra of both viruses, two proteins with a molecular weight of 54,000 and 28,000 were detected. Fractionation of alkali treated polyhedra resulted in: polyhedral membrane, a virus particle and a solubilized polyhedral protein fraction. The polyhedral membrane fraction of the polyhedra of both NPV gave only one protein band of about 28,000 in polyacrylamide gels. The virus particles of the *A. orana* NPV were singly embedded in the polyhedral matrix and those of the *B. brassicae* NPV multiply embedded. They measure 250 x 60 nm and 347 x 113 nm, respectively. The NPV of *A. orana* gave in polyacrylamide gels 5 proteins of molecular weight: 68,000, 48,000, 39,000, 32 - 34,000 and 28,000. The NPV of *B. brassicae* gave proteins of molecular weight: 69,000, 57,000, 46,000, 34 - 39,000 and 28,000. The polyhedra of both insects treated by alkali (dependent on degradation state) gave protein bands of about 28,000, 26,000, 21,000, 18,000, 11,000 and 8,000.

INTRODUCTION

The use of persistent and broad spectrum insecticides to control pests poses several problems. There is a need to develop alternative pest control methods. Nuclear polyhedrosis and granulosis viruses seem to be the most specific and promising among viral agents for use against susceptible artropod pest populations (Ignoffo, 1967; Ignoffo, 1973; Rollinson et al., 1965; Stairs, 1971). The *A. orana* and the *B. brassicae* NPVs have also been shown to have a considerable potential as biological agents for the control of *A. orana* and *B. brassicae* (Ponsen and De Jong, 1964). Furthermore with a cross-inoculation experiment, Ponsen and De Jong observed that *B. brassicae* polyhedra were pathogenic for *A. orana*.

To investigate further this possibility, it is necessary to identify the polyhedral components. The structure of *A. orana* and of *B. brassicae* polyhedra and virus particles in general has been described by Ponsen et al. (1965) and Ponsen (1965).

The present study aimed at characterizing in more detail the NPV of *A. orana* and of *B. brassicae* by electron microscopy and polyacrylamide gel electrophoresis.

MATERIAL AND METHODS

Purification of polyhedra. To purify the polyhedra, the procedure of Van der Geest (1968) was used. The dead larvae were triturated and after straining through cheese cloth, the polyhedra were separated from cell debris by placing aliquots of the crude extract on a sucrose solution with a density of 1.3 g/ml (61.7% w/w). The polyhedra were accumulated at the interface between sucrose solution and water by centrifugation for 30 min at 8,000*g*. The polyhedra were collected and resuspended in water and then placed on top of a sugar solution with a density of 1.2 g/ml (43.9% w/w). The polyhedra were precipitated by centrifugation at 8,000*g* for 30 min, and stored at $4^{o}C$ after removal of the sucrose by washing.

Dissolution of polyhedra. The method of Summers and Paschke (1970) was modified to dissolve the polyhedra and to separate the polyhedral components. The polyhedral components were isolated from highly purified polyhedra of *A. orana* by dissolving them in 0.03 M sodium carbonate *p*H 10.55 for 30 min and those of *B. brassicae* in 0.015 M sodium carbonate *p*H 10.3 for 15 min at $4^{o}C$. The

reaction was stopped by the addition of 0.1 M sodium phosphate buffer *p*H 7.0 (1 : 4).

Separation of the polyhedral components. The solution of dissolved polyhedra was centrifuged at 5,000*g* for 15 min to separate any undissolved polyhedra and polyhedral membranes (pellet) from virus particles (supernatant). The pellet was resuspended and layered on a linear gradient of 40-70% sucrose, dissolved in 0.05 M sodium phosphate buffer, *p*H 7.8. The polyhedral membranes which sedimented in a band by centrifuging at 23,000 rpm for 1 hr in a SW-25 rotor, were collected and suspended in 0.01 M sodium phosphate buffer *p*H 7.0 and stored at $4^{O}C$. The supernatant containing virus particles and degraded polyhedral proteins was also layered on a linear gradient of 10-40% (w/v) sucrose dissolved in 0.05 M sodium phosphate buffer *p*H 7.8 and centrifuged at 23,000 rpm for 30 to 40 min in a SW-25 rotor. The band of virus particles was suspended in 0.05 M sodium phosphate buffer *p*H 7.8 and centrifuged for 45 min at 35,000 rpm in a SW-50 rotor. The polyhedral proteins were isolated from the top fraction of the sucrose gradient by adjustment with 1 N HCl to *p*H 5.8. The precipitated proteins were collected into a pellet by centrifugation at 3,000*g* for 15 min. The proteins were dissolved in 0.01 M sodium phosphate buffer *p*H 7.0.

Transmission electron microscopy. The virus particles, polyhedral membranes or polyhedra were negatively stained with 2% potassium phosphotungstic acid, *p*H 6.8 for 30 sec. Specimens were observed in a Siemens Elmiskop 101 operating at 60 kV and a magnification of 20,000 to 40,000 x.

Scanning electron microscopy of polyhedra. The wet polyhedra, spread on specimen mounts were allowed to dry. To obtain optimum secondary emission of electrons, the specimens were coated with about 200 Å (total) of carbon, and then with gold while rotating on a 'wabble' stage in a vacuum evaporator. The polyhedra were observed with a scanning electron microscope at 10,000 times magnification, operated at an accelerating voltage of 15 kV.

Estimation of molecular weight of proteins by electrophoresis. To prepare the gels the method of Weber and Osborn (1969) was followed. Gel buffer contained 7.8 g $NaH_2PO_4.H_2O$, 38.6 g $Na_2HPO_4.7\ H_2O$ and 2 g sodium dodecyl sulphate (SDS) per litre. An acryl/bis solution was prepared by dissolving 22.2 g acrylamide and 0.6 g methylenebisacrylamide in water to give 100 ml solution. To prepare 8 gels, 7.5 ml gel buffer was mixed with 6.77 ml acryl/bis solution, 22.5 μl tetramethylethylenediamine and 750 μl freshly prepared ammonium persulphate solution (15 mg/ml). After mixing, each tube was filled with 2 ml of the gel solution. A few drops of water were layered onto the gel solution.

The marker proteins used were cytochrome C (Calbiochem, mol wt 11,300), ribonuclease (Sigma, mol wt 13,700), ovalbumin-bovine (Sigma, mol wt 43,000), carboxypeptidase A (Worthington, mol wt 34,000), phosphorylase a (Sigma, mol wt 92,500), trypsin (Sigma, mol wt 23,300), catalase (Sigma, mol wt 60,000). The marker proteins and proteins to be studied were treated by boiling for 2 min in a mixture of 0.01 M sodium phosphate buffer, pH 7.0, 2% SDS and 1% β-mercaptoethanol (Maizel, 1971). Before electrophoresis, 50 μl of by boiling treated protein solutions were mixed with 3 μl 0.05% bromphenol blue in water, 1 drop glycerol and 5 μl β-mercaptoethanol. Electrophoresis was performed at a constant current of 8 mA per gel for 3½ hr. Gels were stained for 2 hr in a solution of 1.25 g Coomassie brilliant blue R 250 dissolved in a mixture of 454 ml 50% methanol and 46 ml glacial acetic acid. The gels were destained with a solution containing 75 ml acetic acid, 50 ml methanol and 875 ml water. The gels were scanned at 550 nm with a Beckman DU spectrophotometer. The distance the marker proteins migrated was plotted against the logarithm of their known molecular weights. The molecular weight of the polyhedral and of the virus proteins was estimated from the distance they migrated (Weber and Osborn, 1969).

RESULTS

Scanning electron microscopic observations on polyhedra

Observations indicated that the polyhedra of *A. orana* and of *B. brassicae* form a heterogeneous population in size and shape. The diameter of *A. orana* polyhedra ranged from 1 to 2 μm and those of *B. brassicae* from 1.5 to 4 μm. Most of *A. orana* polyhedra had a globular shape with some protrusions (Fig. 1). Most of *B. brassicae* polyhedra were hexagonal or pentagonal in outline (Fig. 2). In polyhedral preparations of both species extremely large and differently shaped polyhedra were observed (Fig. 3). As seen on Figs. 1 and 2, some polyhedra had longitudinal cavities on the surface. The cavities on the surface of the *A. orana* polyhedra ranged in length from 0.2 to 0.3 μm and those on the surface of the *B. brassicae* polyhedra ranged in size from 0.3 to 0.4 μm.

Electron microscopic observations on the polyhedral components

The polyhedral membranes were isolated from the pellet obtained after low-speed centrifugation of polyhedra treated by alkali. By centrifuging on a

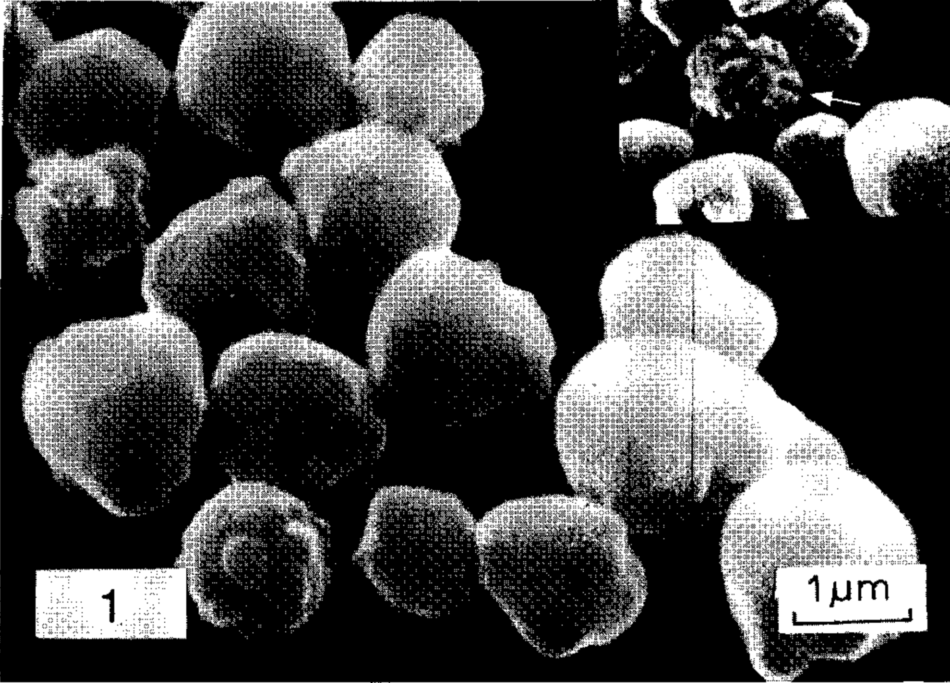


Fig. 1. Polyhedra of *Adoxophyes orana*. Cavities are present on the surface of the smaller polyhedra (upper right).

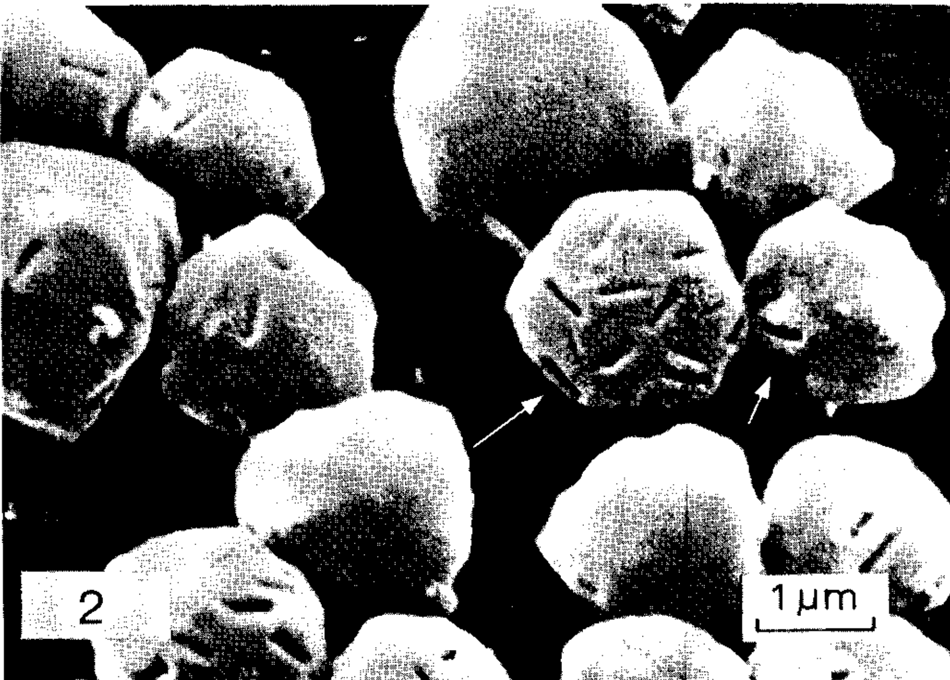


Fig. 2. Polyhedra of *Barathra brassicae* with longitudinal cavities on the surface.

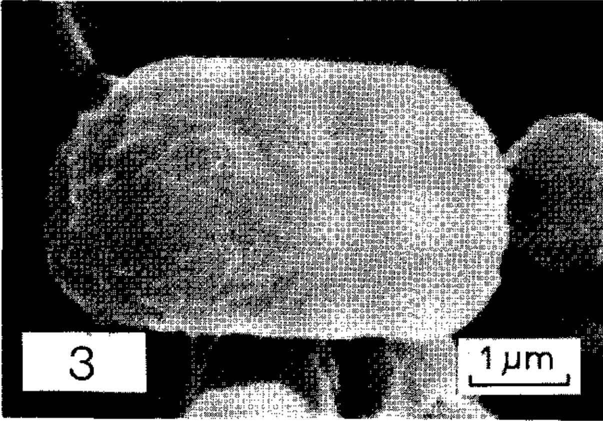
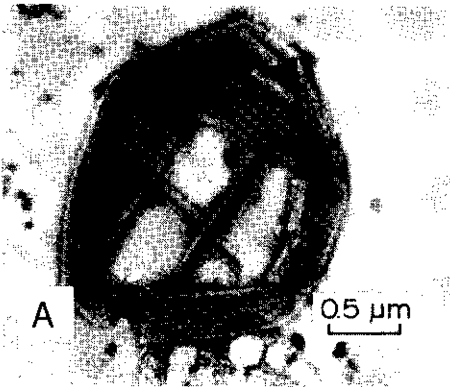


Fig. 3. A polyhedron of *Barathra brassicae*. This polyhedron differs from usual polyhedra in size and shape.



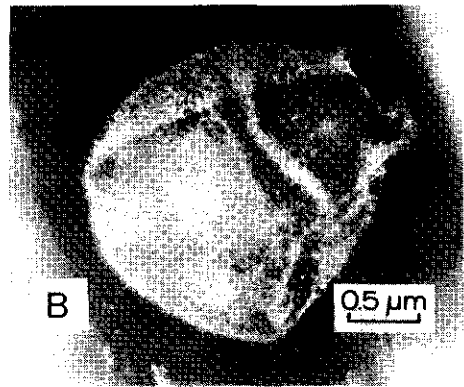


Fig. 4. Polyhedral membrane of the *B. brassicae* polyhedra with a few virus particles (A), polyhedral membrane of the *A. orana* polyhedra without virus particles (B) and pieces of membranes of the *A. orana* polyhedra (C).

40-70% sucrose gradient the membranes formed a band whereas the partially degraded or undegraded polyhedra sedimented to the bottom of the tubes. As seen in Fig. 4, the membranes which sedimented in a band contained a few virus particles (A) or they were completely empty (B). About 40% membranes of the *A. orana* polyhedra and about 60% membranes of the *B. brassicae* polyhedra were broken in pieces (C). The membranes were also observed on the partially degraded polyhedra from the bottom fraction (Fig. 5).

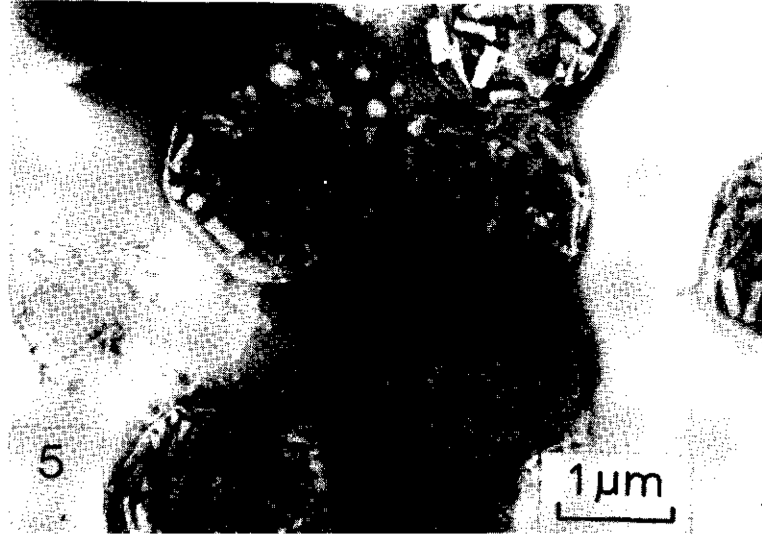


Fig. 5. Partially degraded polyhedra of *B. brassicae* found in bottom fraction after gradient centrifugation of the pellet obtained by low-speed centrifugation of polyhedra treated with 0.015 M sodium carbonate for 15 min at 4°.

The virus particles were isolated from the supernatant obtained after low--speed centrifugation of polyhedra treated by alkali. By centrifuging on a 10-40% sucrose gradient the virus particles were separated from alkali solubilized polyhedral proteins. The virus particles of *A. orana* formed 1 or 2 discrete bands and those of *B. brassicae* 4 or 5 bands in the lower half of the gradient whereas the polyhedral proteins formed a diffuse band near the top of the gradient. Every virus band contained a mixture of virus particles in a different state of degradation. The heavier band contained a lower number of damaged virus particles than the lighter band. In general the lighter band of the *A. orana* NPV contained about 50% of complete particles and about 50% damaged particles. The heavier band contained about 90% complete particles and about 10% damaged particles. The difference in quality of virus particles from the lighter and the heavier band can be seen in Fig. 6. The lightest band of

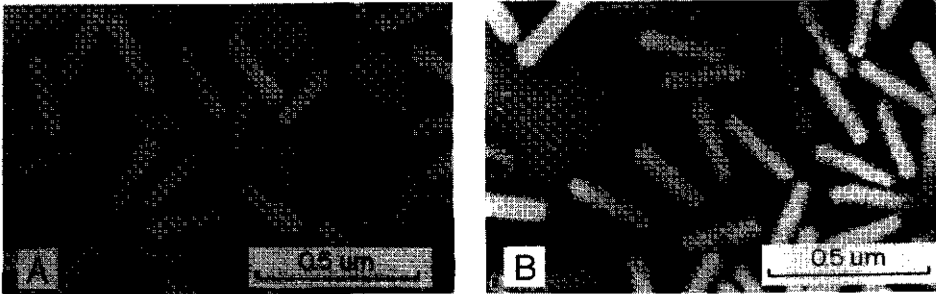


Fig. 6. Nuclear polyhedrosis virus of *A. orana* purified on 10-40% sucrose gradient. The virus particles of the lighter band (A) and the particles of the heavier band (B).

the *B. brassicae* NPV contained only about 5% complete particles and about 95% damaged particles. The heaviest band contained about 70% of complete virus particles. The difference in quality of the *B. brassicae* NPV particles between the lightest and heaviest band can be seen in Fig. 7. Almost all particles of

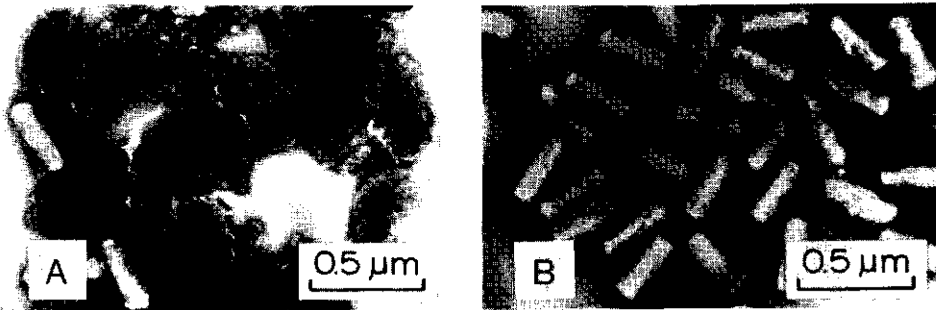


Fig. 7. The NPV of *B. brassicae* purified on 10-40% sucrose gradient. Virus particles of the lightest band showing a predominance of damaged particles (A) and virus particles of the heaviest band showing a predominance of complete particles (B).

the *A. orana* NPV were enveloped singly but occasionally two virus particles were found within an outer membrane. *B. brassicae* NPV particles were usually enveloped in groups but occasionally single virus particles were observed (Figs. 8 and 9, details). The size of the virus particles varied when measured in partially degraded polyhedra (Figs. 8 and 9). Those of *A. orana* were 250 nm long and 60 nm wide and those of *B. brassicae* were 347 nm long and 113 nm wide. The length measurements are given in Figs. 10 and 11. The size of virus particles was about 5% larger when measured on micrographs of virus particles purified on a sugar

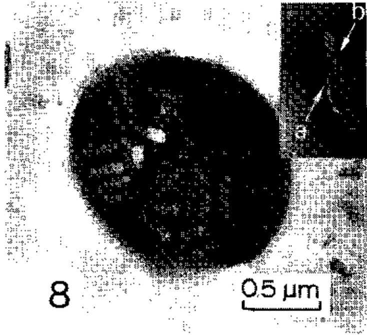


Fig. 8. Partially degraded polyhedra of the *A. orana*, with clearly visible virus particles which were used for measurement of length. Upper right: detail of a single embedded virus particle. Outer membrane (a), inner membrane (b).

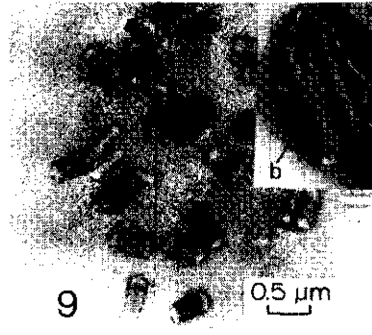


Fig. 9. Virus particles in partially degraded polyhedra of *B. brassicae* which were measured. Upper right: detail of multiple embedded virus particles. Outer membrane (a), inner membrane (b).

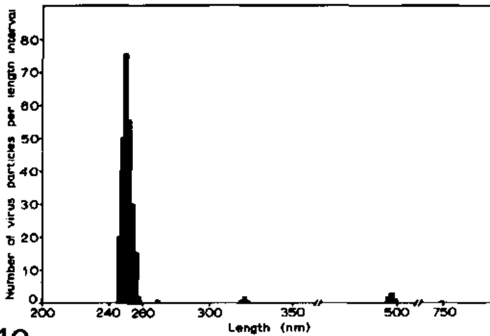


Fig. 10. Histogram of the length of virus particles of the *A. orana* NPV.
258 virus particles were measured.

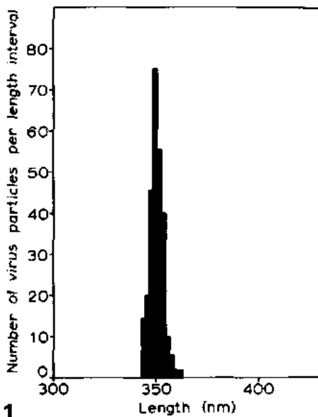


Fig. 11. Histogram of the length of virus particles of the *B. brassicae* NPV.
269 virus particles were measured.

gradient. The virus particles extracted from polyhedra of *A. orana* and of *B. brassicae* by alkali treatment were surrounded by two membranes. The outer membrane of both viruses had no visible sub-structures when negatively stained. Prolonged incubation of virus particles in sodium carbonate disrupted the membranes. The disruption of outer membranes by "dissolution or breaking" on both viruses started at arbitrary places (Fig. 12). After damage of the outer membrane, the inner

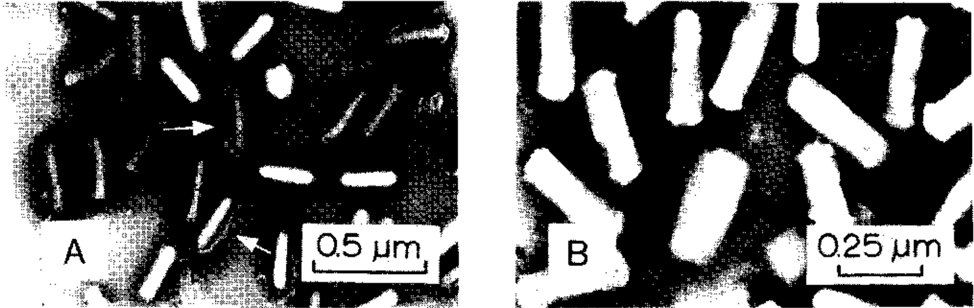


Fig. 12. The disruption of the outer membrane of the *A. orana* NPV by "breaking and dissolution" (A) and the disruption of the outer membrane of the *B. brassicae* NPV by "dissolution" (B).

membrane became visible. The inner or intimate membranes (Bergold, 1963) were observed with or without internal content or in a variety of intermediate states. A regular structure was seen on negatively stained inner membranes with the internal content, but not on inner membranes without internal content. The empty inner membranes were transparent (Fig. 13). A tube was sometimes seen emerging

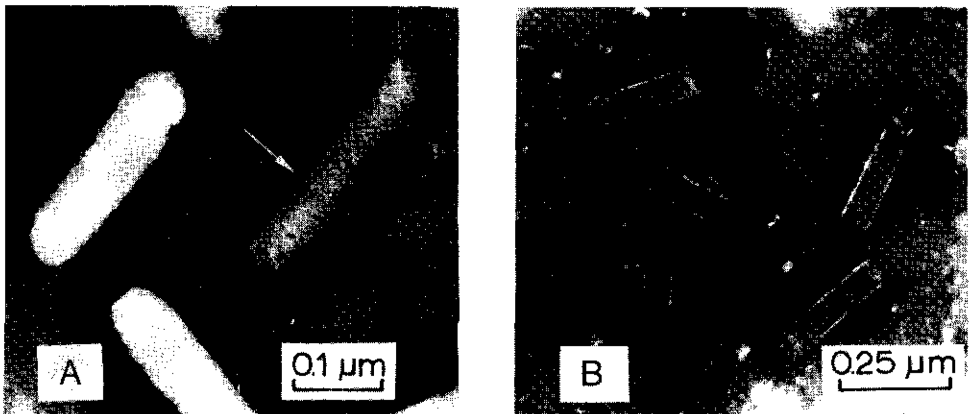


Fig. 13. An inner membrane of the *A. orana* NPV with the internal content (A) and without internal content (B).

from disintegrating virus particles. On the top of these tubes strands believed to be nucleic acid, were observed (Fig. 14).

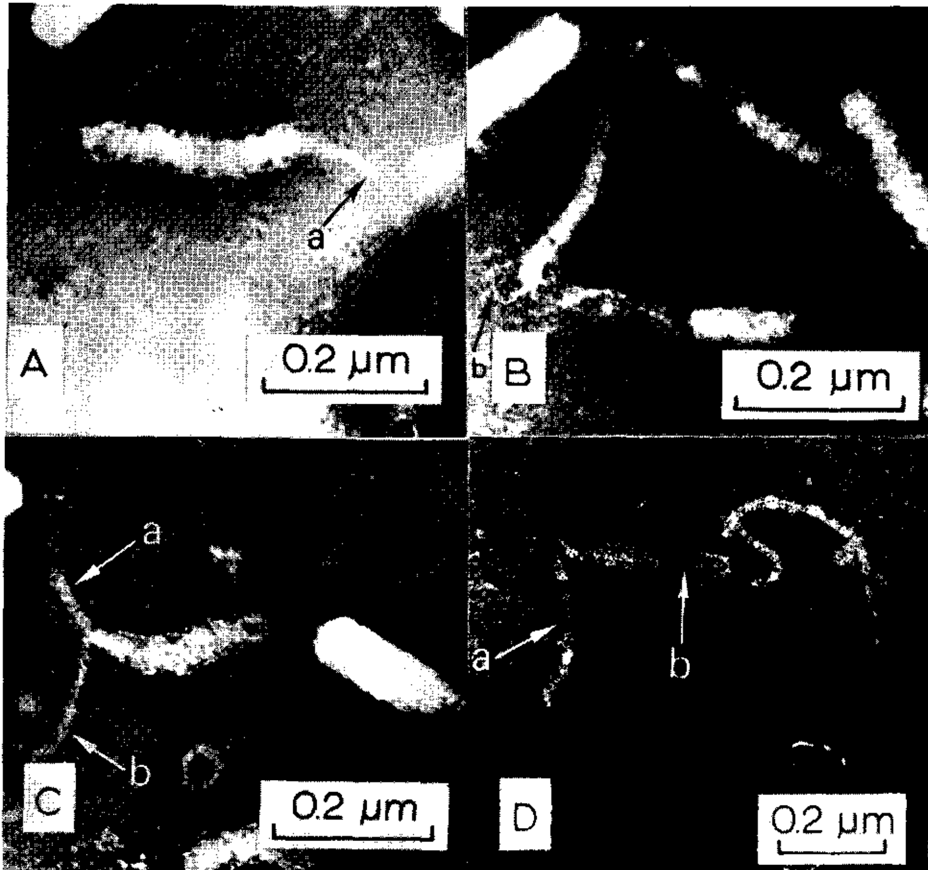


Fig. 14 A,B,C,D. Tube-like structures and strands releasing from inner membranes of the *A. orana* NPV. Tube-like structures (a) and strands (b) are indicated by arrows.

Identification of proteins of the polyhedra and polyhedral components in polyacrylamide gels

The polyhedra of *A. orana* and of *B. brassicae* suspended in 0.01 M sodium phosphate buffer, 2% SDS and 1% β-mercaptoethanol treated at 100°C for 2 min gave two polypeptide bands after electrophoresis in polyacrylamide gels. These proteins of molecular weight 54,000 and 28,000 have been designated as polyhedral protein (PP) I and II. Sometimes a protein of about 70,000 designated as PP A was observed. The ratio of both proteins varied in the different batches of polyhedra purified from *A. orana* and from *B. brassicae* larvae. However the polyhedra of *B. brassicae* always contained more PP I than II.

Differential low-speed centrifugation of the *A. orana* polyhedra showed that the relative amount of polyhedral proteins also varied within a single batch. The

polyhedra obtained by centrifugation of a sample for 10 min at 3,000*g* (Pellet 1) and those obtained by a further centrifugation of the supernatant (Pellet 2) were analysed by electrophoresis. The results show that the polyhedra of Pellet 1 contained more PP I than PP II, and that the polyhedra of Pellet 2 contained more PP II and less PP I (Fig. 15). The polyhedra of Pellet 1 (most of them were surrounded by a membrane) had a somewhat larger diameter than those of Pellet 2 (about 25% of them were not surrounded by a membrane).

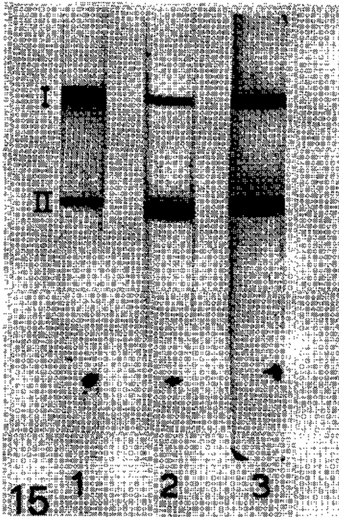


Fig. 15. Electrophoretic pattern of *A. orana* polyhedra. Pattern of the polyhedral pellet obtained by centrifuging a single polyhedral batch at 3,000*g* for 10 min (1). Pattern of the polyhedral pellet obtained by prolonged centrifugation of supernatant (2). Pattern of the unfractionated polyhedral batch (3).

The polyhedral proteins of both NPVs obtained by dissolution of polyhedra with alkali gave in polyacrylamide gels five or six protein bands (designated 1-6) of molecular weight of about 28,000, 26,000, 21,000, 18,000, 12,000 and about 10,000, when the proteins were purified from alkali-treated polyhedra as described in materials and methods. The relative quantity and the number of polypeptide bands that arose due to alkali treatment, depend on the duration of the treatment (Figs. 16 and 17).

The highly purified fraction of polyhedral membranes of both polyhedra gave only one polypeptide band of about 28,000 in polyacrylamide gels.

The virus particles of the *A. orana* NPV gave a pattern with four or five bands in 10% polyacrylamide gels, designated as virus proteins (VP) I-IV and x. When virus particles, obtained after 30 min incubation of polyhedra in alkali (Fig. 18-A), were used for polyacrylamide gel analyses, five bands were observed

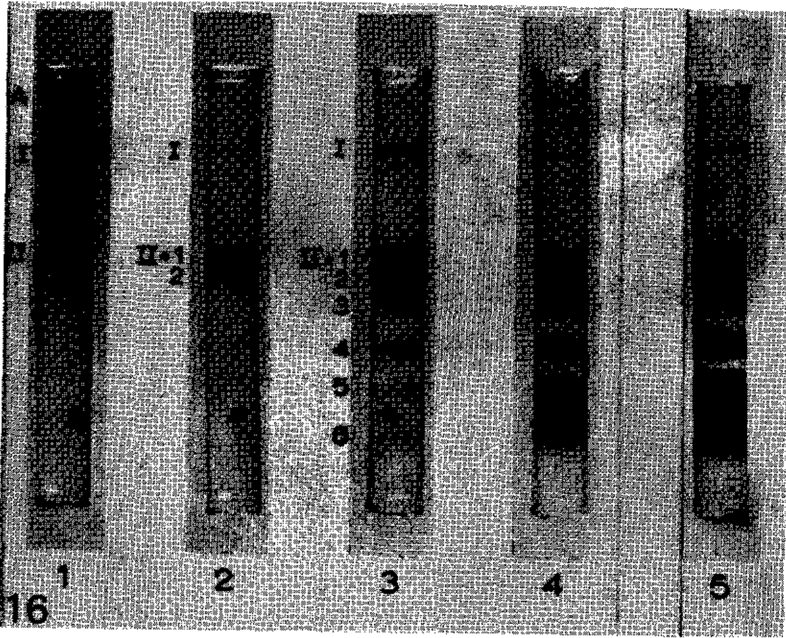


Fig. 16. Electrophoretic pattern of by alkali solubilized *A. orana* polyhedral proteins. Pattern of polyhedra before alkali treatment (1), patterns of polyhedral proteins obtained after incubation of polyhedra in alkali for: 10 min (2), 30 min (3), 45 min (4) and 60 min (5).

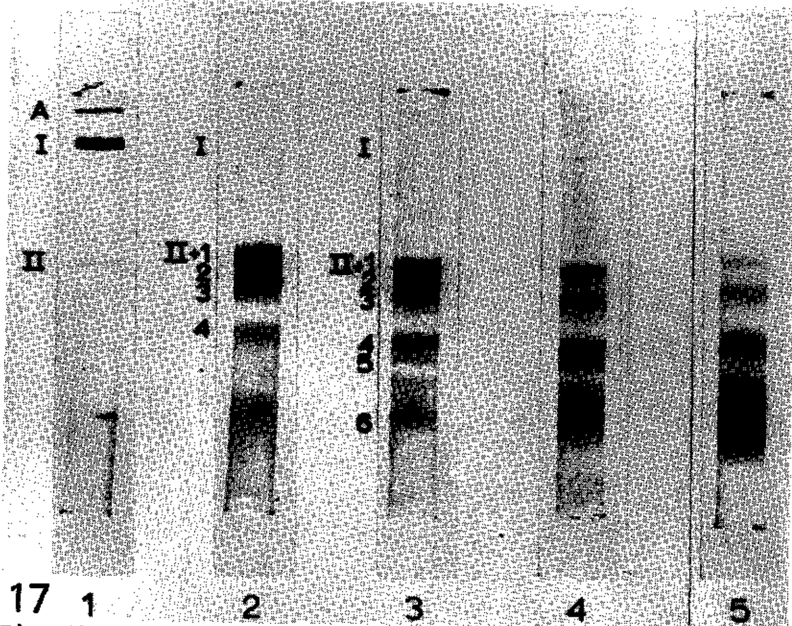


Fig. 17. Electrophoretic pattern of by alkali solubilized *B. brassicae* polyhedral proteins. Undissolved polyhedra (1), patterns of proteins obtained after incubation of polyhedra in alkali for: 5 min (2), 10 min (3), 20 min (4) and 30 min (5).

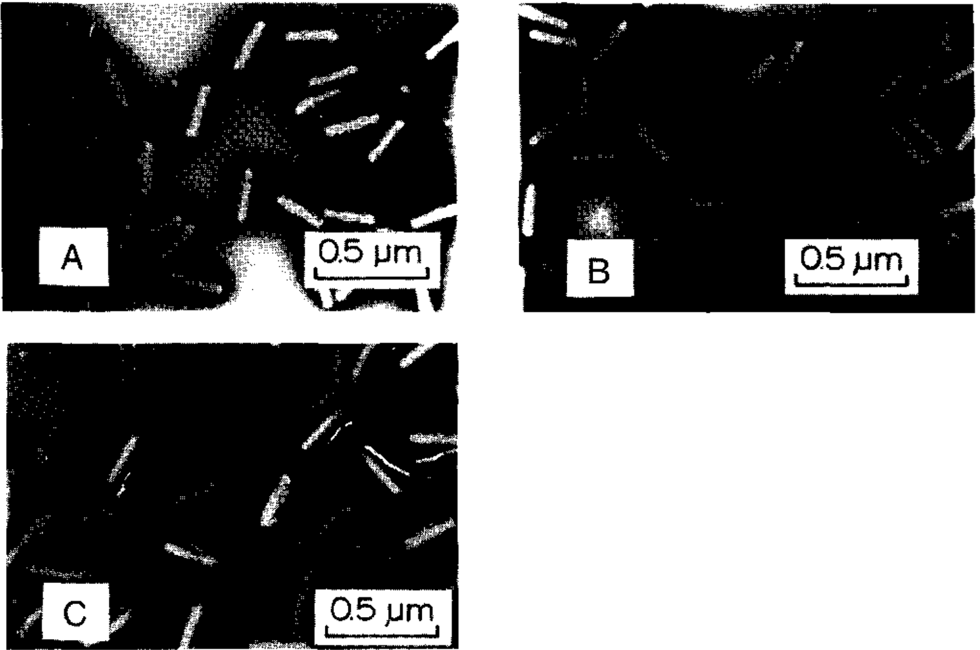


Fig. 18. The *A. orana* NPV. Virus particles with bits of matrix protein (A), virus particles without bits of matrix protein, some particles degraded (B) and virus particles with degraded outer and inner membranes (C). The virus particles were obtained after dissolution of polyhedra in alkali for 30, 45 and 90 min, respectively.

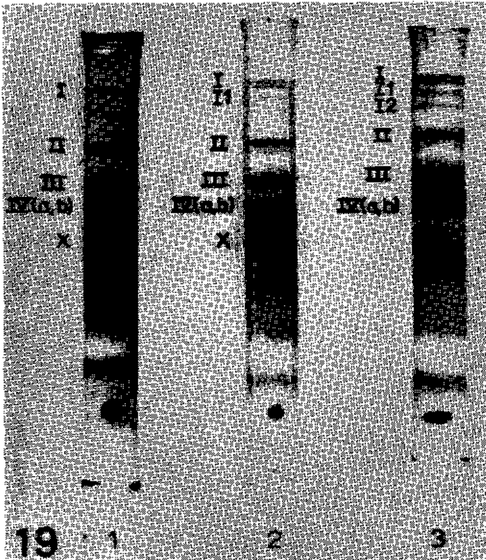


Fig. 19. Electrophoretic patterns of the *A. orana* NPV obtained after dissolution of polyhedra in alkali for: 30 min (1), 45 min (2) and 90 min (3). Bands I_1 and I_2 are the degradation products of band I.

as well as some unspecified bands in the low molecular region (Fig. 19-1). When the virus particles, obtained after 45 min incubation of polyhedra in alkali (Fig. 18-B), were electrophoretically analysed, five distinct bands could also be seen. The band of VP x was much smaller and that of VP 1 disintegrated into bands of lower molecular weight (Fig. 19-2). When virus particles, obtained after 90 min incubation of polyhedra in alkali, were analysed by electrophoresis (Fig. 18-C), only four polypeptide bands were observed. Band of VP x disappeared and that of VP I continued its degradation in I_1 and I_2 (Fig. 19-3). The proteins of the *B. brassicae* NPV gave the same degradation pattern in gels as those of the *A. orana* NPV except that when VP x disappeared VP II was degraded. Besides the electrophoretic patterns, the molecular weights of virus proteins were estimated. The proteins of the *A. orana* NPV had a molecular weight of 68,000, 48,000, 39,000 and 32 - 34,000 (one band with one shoulder) and those of the *B. brassicae* NPV 69,000, 57,000, 46,000 and 34 - 39,000 (one band with two shoulders). The protein band of 28,000 was observed for both viruses.

DISCUSSION

Polyhedra

The cavities on the surface of *A. orana* NPV polyhedra had a length of 0.2-0.3 μm and those on *B. brassicae* NPV polyhedra had a length of 0.3-0.4 μm, therefore it is suggested that the virus particles are embedded in them (Figs. 1 and 2). Selected micrographs showed that small polyhedra in early stages of development had more cavities on the surface than the bigger ones in later stages of development. Cross-sections of polyhedra demonstrated that the cavities were mostly on the surface of those polyhedra which were not, or only partially, surrounded by a membrane. Comparative study by polyacrylamide gel analysis showed that polyhedra of both insects usually give 2 protein bands in polyacrylamide gels. Since 2-5% of the polyhedra are virus particles (Bergold, 1953a), it is reasonable to suppose that PP I and II obtained in polyacrylamide gels after treatment of polyhedra with SDS are polyhedral proteins. The result of the low-speed centrifugation experiment (Fig. 15) and the amount of PP II in general suggest that PP II must be found in the matrix as well as in the polyhedral membrane. However, it is not yet known whether PP I is a higher polymer of PP II because the polyhedral components have not as yet been separated without alkali treatment and further characterized.

Polyhedral proteins obtained by alkali treatment of polyhedra

As discussed earlier the whole polyhedra, treated with SDS gave 2 protein bands in acrylamide gels (PP I 54,000 and PP II 28,000). The electrophoretic pattern of polyhedral proteins of *A. orana* and of *B. brassicae* polyhedra changed when the polyhedra were solubilized in sodium carbonate, isolated and then treated with SDS and analysed by electrophoresis. As seen in Figs. 16 and 17, the PP I of both polyhedra disappeared very quickly under alkaline conditions. However, PP I of *B. brassicae* polyhedra (at a lower salt concentration and a lower *p*H) degraded much faster than that of *A. orana* polyhedra.

Polyhedral membranes

During the sodium carbonate treatment of polyhedra, the electron dense outer layer became detached (Fig. 5). Many scientists have observed these layers and referred to them as membranes (Bergold, 1951; Smith, 1967; Benz, 1963; Teakle, 1967; Harrap, 1971). However, for example Arnott and Smith (1968) suggested that the outer layer is an artifact of preparative processes. In agreement with Ponsen et al. (1965) this study showed that the polyhedral membranes of *A. orana* and of *B. brassicae* polyhedra are structural entities and can be obtained in a highly purified state by purification on a sucrose gradient (Fig. 14). Electrophoresis of polyhedral membranes, disrupted with SDS gave only one protein band of a molecular weight of 28,000. It is the same mol wt as that of PP II, obtained after electrophoresis of polyhedra (undegraded by alkali). However, it has not yet been demonstrated whether the polyhedral membrane protein and the PP II are really the same protein. It is also impossible to determine whether PP I is present in the polyhedral membrane because the polyhedral membranes must be purified with alkali which dissolves PP I too quickly.

Virus particles

The present study shows that the virus particles of both NPVs are labile under alkaline conditions (Fig. 18). However the virus particles of the *B. brassicae* NPV are more labile than those of the *A. orana* NPV. During alkali treatment virus particles began to disintegrate when they were still inside polyhedra. This observation contradicts results of Lewandowski and Traynor (1972). Negatively stained preparations showed that viruses released from polyhedra by alkali treatment could be totally enveloped whereas others were partially or completely devoid of outer membrane. As seen in Fig. 12, the outer membrane was destroyed by "dissolution" which began at arbitrary places or by

combination of dissolution and loss of outer membrane. Negative staining cannot be responsible for the variation in structure observed, because after prolonged alkali treatment more and more disrupted particles were observed. When the outer virus membrane was damaged, the inner membrane appeared. It is not understood why some inner membranes, still partially surrounded by an outer membrane, became longer (or broke) and why some inner membranes without protection of outer membranes (completely empty) were of normal size. The tube-like structures as seen in Fig. 14 were released from the inner membranes, except at the top of these tubes where a string (released or unfolded from these tubes) which is most probably DNA, was observed. These observations indicate that the membranes do not directly enclose the virus nucleic acid. It is likely that the tubes are an external component of the nucleocapsid and are released when the inner membrane is disrupted or removed. Tubes observed in this study were much longer than those observed by Ponsen et al. (1965). They were also much longer than the virus particles and therefore are probably coiled inside the inner membrane. The regular structure, which was seen by electron microscopy on inner membranes with the internal content, could also be due to the coiled form of these tube-like structures. This idea was also supported by the observations on inner membranes without internal content which were transparent (Fig. 13). Monsarrat et al., 1975 and Payne et al., 1977, also concluded that "tails" (tube-like structures) were arranged within the nucleocapsid and may represent nucleoprotein cores of other Baculoviruses. The degradation of virus particles (Fig. 18) resulted in the change of their electrophoretic pattern (Fig. 19). The degradation of virus particles by prolonged incubation in sodium carbonate was also used to suggest the topography of some proteins within the virus particles. It was concluded that VP I of the *A. orana* NPV, VP II of the *B. brassicae* NPV and VP x of both NPVs must be found in the outer membrane. The fact that VP x is present in the outer membrane and has the same molecular weight as PP II of the polyhedra and the same as membrane protein, makes VP x extremely interesting. It is important to study the nature of VP x and of the other polyhedral proteins, so that it can be determined whether VP x is really the same protein as PP II of the polyhedra and as polyhedral membrane protein. If all these proteins are the same, the second important task is to determine their origin, because we are not certain whether the polyhedral proteins are coded for by the virus or the host (Payne et al., 1977). Thus there are two possibilities: first, if these proteins are the same and they are coded for by the virus, then VP x would be the major component of an "outer virus membrane" which is a structural entity of a virus. Here VP x

probably serves as a nucleation site on which the protein crystal that forms the polyhedron develops; second, if all these proteins are the same and they are coded by host genome, then VP x would not be a component of the outer virus membrane, but would form a layer with a specific affinity for the virus membrane. Only when this problem is solved, can we prove whether the NPV of *A. orana* and of *B. brassicae* are really surrounded by two virus membranes as suggested by Ponsen et al. (1965) and by other workers for other NPVs, since the discovery of Bergold (1953a, b, c).

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II EFFECT OF AN ALKALINE PROTEASE ASSOCIATED WITH THE NUCLEAR POLYHEDRA OF *ADOXOPHYES ORANA* AND OF *BARATHRA BRASSICAE* ON THEIR SOLUBILIZATION

SUMMARY

The polyhedra of the *Adoxophyes orana* and of the *Barathra brassicae* nuclear polyhedrosis virus, without alkali treatment ("whole" polyhedra), gave peptide bands of about 28,000 and 54,000 in polyacrylamide gels. Effect of an alkaline protease on the solubilization of polyhedra was demonstrated by comparison of electrophoretic pattern of proteins obtained after alkali treatment of "enzyme-inactivated" polyhedra with that of "enzyme-active" polyhedra. Electrophoretic pattern of "solubilized enzyme-inactivated" polyhedra differed from that of "whole" polyhedra. The polyhedral preparation also gave two peptides but of molecular weight 28,000 and 26,000. Electrophoretic pattern of "solubilized enzyme-active" polyhedra differed from that of "whole" polyhedra and also from that of "solubilized enzyme-inactivated" polyhedra. The polyhedral preparation gave a mixture of at least six peptides with molecular weight between 28,000 and 8,000. Results indicated that the solubilization of polyhedra can occur in two steps: proteolytic enzyme independent and proteolytic enzyme dependent.

INTRODUCTION

Since Bergold (1947) described solubilization of polyhedra by weak alkali most researchers routinely "dissolve" polyhedra by treating them with various concentrations of sodium carbonate or sodium carbonate with NaCl for different lengths of time (Smith, 1976). Increasing the period of exposure to sodium carbonate caused a release of virus particles, polyhedral membranes and solubilization of polyhedral proteins. The nature of this phenomenon is unknown at present. But since the many reports about the association of proteinases with polyhedra (Eppstein and Thoma, 1975; Eppstein et al., 1975; Kozlov et al., 1975; Summers and Smith, 1975; Payne and Kalmakoff, 1978) solubilization of polyhedra by alkali has become a highly intriguing problem. Because, if the viruses and polyhedral proteins are nonspecifically destroyed, modified or removed artifactually during the solubilization of polyhedra, it would be impossible to detect significant differences between the polyhedral viruses.

This report discusses the effect of an alkaline protease on the solubilization of *Adoxophyes orana* and of *Barathra brassicae* polyhedra. Furthermore it puts forward evidence of the possible origin of an alkaline protease.

MATERIALS AND METHODS

Production and purification of polyhedra. Fourth instar larvae of *A. orana* and of *B. brassicae* insects were fed on foliage previously coated with a suspension of polyhedra of the homologous insects. Twenty days after inoculation, NPV--diseased larvae were triturated and after straining of the macerate through cheese-cloth the polyhedra were separated from cell debris (Van der Geest, 1968). Aliquots of the crude extract were placed on top of sucrose solution 61.7% (w/w) and centrifuged at 8,000*g* for 30 min. The polyhedra that accumulated at the boundary between the sucrose solution and water were, after collection and resuspension, placed on the top of a sucrose solution 43.9% (w/w). The polyhedra were sedimented by centrifuging at 8,000*g* for 30 min, then washed to remove the sucrose and stored at 4°C. During storage, polyhedra (10 mg/ml) were washed by centrifugation once a month. The supernatants obtained after centrifugation of polyhedral suspensions were used for demonstration of proteolytic activity (PA). The pelleted polyhedra were resuspended in fresh water and further stored.

Preparation of larvae extracts. Healthy or diseased 4th instar larvae were dissected and the guts including digestive fluids and fat body were triturated.

The extracts were separated from crude cell debris by straining through cheese-cloth. These extracts were centrifuged at 3,000*g* for 20 min and then twice at 90,000*g* for 2 hr to remove the remaining cell debris and, if present, polyhedra and virus particles. All treatments were carried out at $4^{o}C$ and the extracts were directly used for assay of PA. Before determination of PA, the extracts were diluted 100 times with sodium carbonate buffer, *p*H 10.5. The final molarity was 0.1.

Inactivation of proteolytic activity. The enzyme was inactivated by heating the polyhedral suspension to $70^{o}C$ for 30 min (Summers and Smith, 1975). The heated polyhedra were pelleted by centrifuging at 5,000*g* for 30 min and as soon as possible used for determination of PA or for isolation of polyhedral proteins.

Determination of proteolytic enzyme activity. The determination of PA was based upon the determination of the casein-split products soluble in perchloric acid. Polyhedra, extracts from larvae and supernatants obtained after washing of polyhedra were assayed for the PA. Standard polyhedral samples (10 ml) were obtained by mixing 5 ml polyhedra suspended in water (1 - 2 mg/ml), 4 ml casein solution (12.5 mg/ml) and 1 ml sodium carbonate buffer (1 M) *p*H 10.5. The extracts from larvae and the supernatants after washing of polyhedra were diluted 100 and 5 - 100 times, respectively, with sodium carbonate buffer before PA assay. The standard final concentrations were: 5 mg/ml casein, 0.1 M carbonate buffer, *p*H 10.5. PA was assayed by incubating the samples at $30 \pm 0.2^{o}C$ in a shaking water bath (100 strokes/min). Samples (volume of 2 ml) were withdrawn at different intervals. The sample withdrawn after 30 sec of incubation served as control. The reactions were stopped by the addition of 0.1 ml of 70% perchloric acid to the samples. After the samples had been centrifuged at 38,000*g* for 20 min, the supernatants were analysed for split products by a modification of the method of Lowry et al. (1951) as described by Herbert et al. (1971). The proteolytic activity was defined as initial increase of casein-split products soluble in perchloric acid, expressed as mg casein per hr per mg of polyhedra or per ml of undiluted larvae extracts (or per ml of supernatants obtained after washing of polyhedra). No correction was made for the degradation of polyhedra's own protein. The expression of PA in mg of casein is not arbitrary since it was shown that the extinction coefficient of the undegraded casein is the same as that of partly degraded casein.

Isolation of polyhedral proteins after solubilization of polyhedra by alkali. Polyhedra of *A. orana* (heated and unheated) were suspended in 0.03 M sodium carbonate *p*H 10.5 to a concentration of 10 mg/ml and incubated at $18^{o}C$. Samples

were taken from the polyhedra suspensions that were not treated with alkali and from polyhedra after 15, 30, 60 min of incubation time in alkali. *B. brassicae* polyhedra (heated and unheated) were suspended in 0.015 M sodium carbonate *p*H 10.3 to a concentration of 10 mg/ml and incubated at 18°C. Samples were taken from polyhedra that were not treated with alkali and from polyhedra after 10, 20, 30 min of incubation time under alkaline conditions. Polyhedra which were not treated with alkali served as control. Reactions were stopped by cooling and by diluting with 0.1 M sodium phosphate buffer (*p*H 7.0) in a proportion 1 : 2. After centrifugation at 90,000*g* for 45 min (to remove the virus particles), the preparations were adjusted to *p*H 5.8 and incubated at 4°C for 16 hr. The flocculated proteins were sedimented by centrifuging at 8,000*g* for 20 min (4°C). The sedimented proteins were dissolved in 0.01 M sodium phosphate buffer, *p*H 7.0 to an optical density of 2 at 280 nm and used for electrophoresis.

Acrylamide gel electrophoresis. To analyse the polyhedral proteins the method of Weber and Osborn (1969) was followed. For a run of 8 gels, 6.77 ml acryl/bis solution (22.2 g acrylamide and 0.6 g methylene bisacrylamide filled up with water to 100 ml), 22.5 µl tetramethylethylenediamine and 750 µl freshly prepared ammonium persulphate solution (15 mg/ml) were mixed with 7.5 ml gel buffer, consisting of: 7.8 g $NaH_2PO_4.H_2O$, 38.6 g $Na_2HPO_4.7H_2O$ and 2 g sodium dodecyl sulphate (SDS) per litre. Cytochrome C (Calbiochem., mol wt 11,300), ribonuclease (Sigma, mol wt 13,700), ovalbumin-bovine (Sigma, mol wt 43,000), carboxypeptidase A (Worthington, mol wt 34,000), phosphorylase (Sigma, mol wt 92,500), trypsin (Sigma, mol wt 23,300) and catalase (Sigma, mol wt 60,000) were used as marker proteins. The marker proteins and proteins to be studied were diluted in a mixture of 0.01 M sodium phosphate buffer (*p*H 7.0), 2% SDS and 1% β-mercaptoethanol and boiled for 2 min (Maizel, 1971). Prior to electrophoresis, the treated proteins (50 µl) were mixed with 3 µl 0.05% bromphenol blue in water, 1 drop glycerol and 5 µl β-mercaptoethanol. Electrophoresis was performed at a constant current of 8 mA per gel for 3½ hr. Proteins were stained with Coomassie-brilliant blue R-250 (Schwarz/Mann) following the method of Fairbanks et al. (1971). The gels were scanned at 550 nm with a Beckman DU spectrophotometer. The migration distance of the marker proteins was plotted against the logarithm of their known molecular weights. The molecular weight of the polyhedral proteins was estimated from their migration distance (Weber and Osborn, 1969).

RESULTS

Demonstration of an alkaline protease associated with polyhedra

To determine the presence of a protease activated by alkali, the polyhedra of *A. orana* and of *B. brassicae* were suspended in 0.1 M sodium carbonate buffer at *p*H 9.5, 10.5, and 11.5. An increase of PA was detected as *p*H increased. In this study the PA of polyhedra of both insects was determined at *p*H 10.5 because at this *p*H the dilution of polyhedra and extraction of virus were usually done. To prevent differences in results caused by experimental conditions, *A. orana* and *B. brassicae* polyhedra were produced, purified and stored under the same conditions. PA of the polyhedra of both insects was measured under the same experimental conditions. Results shown in Table 1 demonstrate that the PA associated with *B. brassicae* polyhedra was much higher than that of *A. orana* polyhedra. The observations also showed that the supernatant obtained after centrifugation of a "fresh" polyhedral suspension as well as supernatant obtained after centrifugation of an "old" polyhedral suspension contained PA. However, the PA of the supernatants of "old" polyhedra was much lower than that of "fresh" polyhedra.

Effect of an alkaline protease on the solubilization of A. orana and of B. brassicae polyhedra

Comparative study by acrylamide gel electrophoresis showed that the whole polyhedra of both insects when treated with SDS gave 2 protein bands in acrylamide gels: one band of about 54,000 designated as PP (polyhedral protein) I and one of about 28,000 designated as PP II. Sometimes a protein of about 70,000 designated as PP A could be observed (Figs. 1A and 2A).

To indicate the influence of an alkaline protease on solubilization of polyhedra, the polyhedra of both insects were divided into two treatment groups. One group of polyhedra was heated to inactivate the protease activity and the other one was unheated. A part of the polyhedral suspension of both groups (of both insects) was used for measurements of proteolytic activity (Table 1) and the other part was used for a study of solubilization of polyhedra under alkaline conditions by electrophoresis (Figs. 1 and 2). A comparison of electrophoretic patterns of heated and unheated polyhedra after alkali treatment showed that they differ from each other. The heated polyhedra after alkali treatment gave two bands in acrylamide gels: one of about 28,000 designated as PP II + 1 and one of

Table 1. Proteolytic activity associated with *A. orana* and with *B. brassicae* polyhedra

Sample of	Approximate PA (mg casein/hr.mg or ml)	Relative activity	Sample of	Approximate PA (mg casein/hr.mg or ml)	Relative activity
A. orana:			*B. brassicae*:		
"Fresh" polyhedra	7.0	14.0	"Fresh" polyhedra	37.5	12.5
Supernatant of "fresh" polyhedra	2.5	5.0	Supernatant of "fresh" polyhedra	5.5	1.8
"Old" polyhedra	4.5	9.0	"Old" polyhedra	12.5	4.2
Supernatant of "old" polyhedra	0.5	1.0	Supernatant of "old" polyhedra	3.0	1.0
Enzyme inactivated polyhedra	NA[1]		Enzyme inactivated polyhedra	NA[1]	

Polyhedra purified by the method of van der Geest (1968) were suspended in water and stored at 4^oC. Before assay the polyhedral suspensions were centrifuged to separate the polyhedra from supernatant. For measurements of PA the polyhedra and the supernatants were diluted so far that the degradation of casein was roughly linear during the time interval used for the calculation of PA (e.g. 15, 30, 60 min). PA associated with polyhedra was defined as mg casein hydrolysed per mg of polyhedra per hr at 30^oC. No correction was made for the degradation of polyhedra's own protein during the degradation of casein. The "old" polyhedra had been stored for 6 months at 4^oC. The PA of supernatants was defined as mg casein hydrolysed per ml per hr at 30^oC. The results are the average of 2 experiments.
NA[1] = no activity

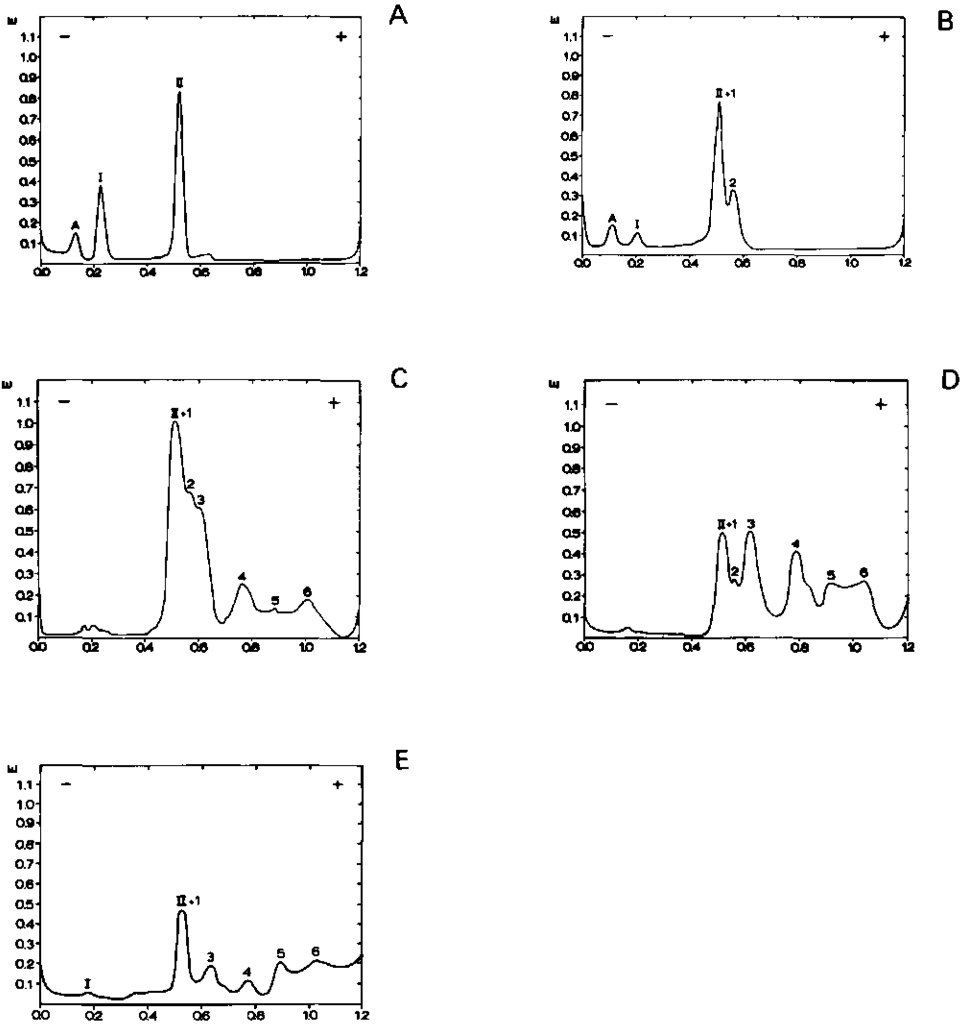

Fig. 1. Densitometric patterns of polyacrylamide gels of *A. orana* polyhedral proteins. Samples of polyhedral proteins obtained from undissolved polyhedra (without alkali treatment) (A), from heated polyhedra, after 60 min of incubation time in 0.03 M sodium carbonate, *p*H 10.5 at a temperature of 18°C (B) and from unheated polyhedra after 15 (C), 30 (D), and 60 min (E) of incubation time in 0.03 M sodium carbonate *p*H 10.5 at a temperature of 18°C. The samples were processed as described in Materials and Methods.

Results shown in Table 2 demonstrate that a high PA was present in extracts prepared from healthy as well as NPV-diseased larvae of both insects. The PA of extracts of healthy larvae was much higher than that of extracts of homologous but NPV-diseased larvae. There was also a clear difference in the level of proteolytic activity between the extracts of *B. brassicae* and of *A. orana* larvae. The highest activity was observed in extracts from healthy *B. brassicae* larvae.

DISCUSSION

The first task of this study was to determine whether an alkaline protease complicated the characterization of proteins of *A. orana* and of *B. brassicae* polyhedra. For this purpose polyhedra not treated with alkali as well as proteins obtained from heated (without active enzyme) and unheated (with active enzyme) polyhedra after alkali treatment were subjected to electrophoresis. The results of this study confirm the results of other workers (Eppstein and Thoma, 1975; Kozlov et al., 1975; Summers and Smith, 1975; Payne and Kalmakoff, 1978, and McCarthy and Shu-Yen Liu, 1976) and they showed that a protease activated by alkaline conditions introduces extensive artifacts into proteins of *A. orana* and of *B. brassicae* polyhedra as analysed by electrophoresis. The polyhedra of both insects, not treated with alkali, gave two polypeptide bands (PP I and PP II) in polyacrylamide gels (Figs. 1A and 2A). However, the electrophoretic pattern of both polyhedra changed when proteins obtained from heated as well as unheated polyhedra after alkali treatment were used for electrophoresis. Furthermore, the electrophoretic pattern of heated polyhedra (without active enzyme) and that of unheated polyhedra (with active enzyme) after alkali treatment differed from each other (Figs. 1B, C and 2B, C). From this difference in electrophoretic patterns, it can be concluded that the polyhedra under alkaline conditions are solubilized in two steps. The first step represents the "solubilization" of PP I into PP 1 (which seems to be associated with PP II) and into PP 2 (Figs. 1B and 2B).
Because this electrophoretic pattern was obtained from heated polyhedra after alkali treatment and this pattern did not change when incubation was prolonged, it was concluded that this step is independent of proteolytic activity. However this step of "solubilization" could also be observed when the proteins of unheated polyhedra after a short alkali treatment were subjected to electrophoresis. The second step of solubilization represents the further solubilization of PP II + 1 and PP 2 into smaller polypeptides (Figs. 1C, D, E). Because this

electrophoretic pattern was obtained only from unheated polyhedra after prolonged incubation in alkali, it is reasonable to suppose that this step is dependent on an alkaline protease.

After the PA associated with polyhedra was detected, the second intriguing task was to determine the origin and the location of this enzyme within the polyhedra. Because proteolytic enzymes have been demonstrated in many insects, and because most insects proteinases have been proved to be active at neutral or alkaline *p*H (Day and Waterhouse, 1953; Gilmour, 1961; Gooding and Huang, 1969; Eguchi et al., 1972; Eguchi and Iwamoto, 1973, and Eguchi and Iwamoto, 1976), the supernatants obtained after centrifugation of polyhedral suspensions and the extracts of healthy as well as NPV-diseased larvae were used for PA assay. The presence of PA in supernatants obtained after centrifugation of polyhedral suspensions (Table 1) suggests that an alkaline protease can be associated with the surface of polyhedra and that this PA can be of host origin. The presence of PA in extracts from healthy and from NPV-diseased larvae suggests that every component of polyhedra, during the formation of polyhedra can be contaminated with one or even more distinct enzymes. Results showed that extracts of healthy larvae had higher PA than those extracts from NPV-diseased larvae (Table 2). However, it is not yet known why the extracts of NPV-diseased larvae had a lower PA than those of healthy larvae. If we assume that the enzyme associated with polyhedra and that in extracts of larvae is the same, then there are two alternative explanations for the lower PA in extracts from NPV-diseased larvae: first, the amount of enzyme produced in diseased larvae is lower than in healthy larvae or second, the amount of enzyme produced by diseased larvae is the same as that produced by healthy larvae but a part of this enzyme could be associated (internal or external) with polyhedra. In contradiction to Eppstein and Thoma (1975), Payne and Kalmakoff (1978) and according to Kozlov et al. (1975) results of this study suggest that PA of polyhedra can arise from insects' own enzymes and can be located externally and internally with polyhedra.

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III CHARACTERIZATION OF THE NUCLEAR POLYHEDROSIS VIRUS DNA OF *ADOXOPHYES ORANA* AND OF *BARATHRA BRASSICAE*

SUMMARY

Circular double-stranded DNA was isolated from nuclear polyhedrosis virus (NPV) of *Adoxophyes orana* (virus particles singly embedded in the polyhedral matrix) and NPV of *Barathra brassicae* (virus particles multiply embedded in the polyhedral matrix) and some of their physical properties were determined. The molecular weights of *A. orana* and *B. brassicae* NPV-DNA, 6.7×10^7 and 8.9×10^7 Daltons, respectively, were determined by electron microscopy and by renaturation kinetics analysis. The latter analysis also showed that both genomes do not contain repetitive sequences. Absence of homology between DNA of these two viruses was shown by competition hybridization of *A. orana* NPV-DNA with *B. brassicae* NPV-DNA. Analysis of these DNAs with the restriction endonuclease *Eco* RI confirmed that they are different. The buoyant densities in CsCl of *A. orana* NPV-DNA and of *B. brassicae* NPV-DNA, 1.694 and 1.696 g/cm^3, respectively, are consistent with (G+C) contents of 34.5 and 37%, respectively, as determined by thermal denaturation.

INTRODUCTION

Since the need to develop alternatives to chemical insecticides is urgent, the potential use of nuclear polyhedrosis viruses as entomo-pathogenic bio--control agents for Lepidoptera has aroused much interest (Summers et al., 1975). Insect viruses, although they seem to be logical substitutes for chemical agents, require fundamental and applied studies, to remove doubts about their utilization. The use of insect viruses as biological agents for insect control and possible hazards have been discussed by the World Health Organization (1973). There are hardly any data available on the genetic material of these biological agents (Davies, 1975). Biochemical and biophysical properties of insect viruses can provide much information but caution is required since the viruses of some invertebrates can be cross-infective (Vail et al., 1973; Beavers and Reed, 1972; Webb et al., 1974; Stairs and Lynn, 1974). Therefore, insect viruses have to be identified and compared mutually as well as with viruses of vertebrates before they can be used for biological control. In our laboratory we are studying the suitability of the nuclear polyhedra of *Adoxophyes orana* and of *Barathra brassicae* for biological control in the field. This article describes part of this work: the characterization and comparison of the genetic material of these two nuclear polyhedrosis viruses.

MATERIALS AND METHODS

Viruses. The sample of the *A. orana* NPV was obtained from Dr. M.B. Ponsen of our laboratory. The *B. brassicae* NPV was obtained from Dr. L.P.S. van der Geest, Laboratory of Entomology, University of Amsterdam. Virus was further produced by inoculation of homologous insect hosts with their own nuclear polyhedrosis virus.

Marker DNAs. DNA of bacteriophage PM2 was provided by Dr. C. Walig, Laboratory for Hygiene, Amsterdam. DNA of Adenovirus Type 5 was obtained from the Laboratory of Physiological Chemistry, Utrecht.

Purification of polyhedra. Polyhedra were purified by differential centrifugation according to the procedure of Van der Geest (1968). Filtered homogenates of NPV-diseased larvae were centrifuged at 3,000g for 30 min at 4°C. The sedimented polyhedra were suspended in distilled water and 10 ml suspension (about 10 mg polyhedra/ml) was layered on 20 ml sucrose solution 61.7% (w/w) in SW-25 rotor tubes. After centrifugation at 8,000g for 30 min at 4°C, the polyhedra

accumulated at the water-sugar interface. The polyhedra were collected and resuspended in distilled water, and the suspension was placed on top of a sucrose solution 43% (w/w). Polyhedra were sedimented by centrifuging at 8,000*g* for 30 min at 4°C. The polyhedra were washed free of sucrose by two cycles of centrifugation at 5,000*g* for 30 min at 4°C. Purified polyhedral suspension was divided into two. One part was stored in distilled water at 4°C and used for purification of DNA for electron microscopy. The second part of polyhedral suspension was lyophylised and used for purification of DNA for thermal denaturation.

Purification of virus. To purify the virus, the method of Summers and Paschke (1970) was modified. The virus particles were extracted from highly purified polyhedra of *A. orana* by dissolving them in 0.03 M Na_2CO_3, *p*H 10.55 for 30 min and those of *B. brassicae* in 0.015 M Na_2CO_3, *p*H 10.3 for 15 min at 4°C. The virus particles were separated from the polyhedral proteins by subsequent centrifugation in a 10-40% sucrose gradient. Centrifuging at 23,000 rpm in a SW-25 rotor for 30 min at 4°C sedimented the virus particles in a band and the polyhedral proteins stayed in the fraction on the top of the gradient. The virus particles were collected with a drop collection unit, Model 195 obtained from ISCO.

Purification of nuclear polyhedrosis virus DNA. A modification of the methods of Gafford and Randal (1967) and Marmur (1961) was used. Virus was suspended in 8 volumes of 0.015 M sodium citrate and 0.15 M NaCl (1 x SSC) *p*H 7.4 plus 10 mM EDTA and 2 volumes of 10% sodium dodecyl sulphate (SDS), after which the suspension was heated in a water bath at 60°C for 30 min. Sufficient 5 M NaCl was added to give a final concentration of 1 M. The suspension was stored at 4°C. After approximately 16 hr, the suspension was centrifuged at 15,000*g* in a Serval RC-2 centrifuge to remove the precipitate. The supernatant was layered onto a solution of CsCl (initial density 1.7 g/cm^3) in SW-50 rotor tubes and centrifuged in a Beckman LB 5-65 preparative ultracentrifuge at 30,000 rpm at 25°C. After 24-36 hr of centrifugation the tubes were fractionated with a drop collection unit Model 195 obtained from ISCO. The DNA fraction was dialysed against 0.02 M Tris-HCl buffer plus 3 mM EDTA, *p*H 8.5.

To increase the quantity of supercoiled DNA, the method described above was shortened by omitting the addition of NaCl and isopycnic centrifugation in CsCl.

Caesium chloride equilibrium centrifugation. The buoyant density measurements were made in a Beckman analytical ultracentrifuge according to Mandel et al. (1968). 10 µl of reference *E. coli* B-DNA or bacteriophage T4 DNA (40 µg/ml) and 40 µl (20 µg/ml) of DNA of a density to be determined were added to 840 µl of a stock solution (13 g CsCl in 7 ml of 0.02 M Tris-HCl buffer *p*H 8.5). The

refractive index of the solution was adjusted with about 200 µl of Tris-HCl buffer to a value of 1.3996. Centrifugation was at 44,770 rpm for 20 hr at 25°C.

Thermal denaturation. Thermal denaturation curves (Tm) were made in 1 x SSC, pH 7.0, according to Mandel and Marmur (1968) in a Gilford Model 2400 spectrophotometer. The DNA samples at a final concentration of 30 µg/ml were dialysed against 1 x SSC, at 4°C, three changes of 16-18 hr each. The DNA preparations did not contain covalently closed DNA as they were prepared from lyophylised virus (Summers and Anderson, 1972b) and twice sheared by Vortex for 15 sec. During melting, the temperature was raised by 0.1°C per min and the absorbance was recorded every minute. The base composition was calculated from Tm values according to Marmur and Doty (1961).

Preparation of DNA samples for electron microscopy. A modification of the Kleinschmidt technique (Kleinschmidt, 1968; Davis et al., 1971) was used. The spreading solution contained: 25 µl 99% distilled formamide, 10 µl water, 10 µl DNA (25 µg/ml), 5 µl 1 M Tris-HCl buffer plus 0.1 M EDTA pH 8.5 and 2.5 µl Cytochrome C (1 mg/ml in 0.02 M Tris-HCl buffer plus 0.002 M EDTA pH 8.5). The mixture was spread on a hypophase containing 20% formamide. A carbon-coated grid was used to collect a small drop from the hypophase surface. The grid was stained with 50 mM uranyl acetate in 90% ethanol for 30 sec, dehydrated in 2-methylbutane for 10 sec and air dried. The grids were rotary shadowed at an angle of 5-7° with platinum-iridium (90-10%).

Electron microscopy of DNA. Electron micrographs were obtained with a Siemens Elmiskop 101 electron microscope, at a magnification of 8-16,000 (80 kV, at 50 µm objective aperture). Magnification was calibrated with a carbon replica of a diffraction grating (2,160 lines per mm). The plates were optically enlarged 4 times by projection. The images were traced on paper and measured by a Hewlett--Packard, Model 10 calculator.

Labelling of DNA for reassociation kinetics. For labelling of DNA, the nick--translation technique described by Rigby et al. (1977) was modified. Isolated DNA was labelled to a high specific radio-activity with DNA Polymerase I, after introduction of single-stranded nicks with DNase I. Reactions were carried out at 13.5°C for 90 min in 50 µl volumes containing 200 p mol (^{32}P) d ATP, 200 p mol (^{32}P) d CTP. The specific activity of the (^{32}P) substrates was 350 Ci/m mole. After evaporation, the following substances were added: 2 µl 0.05 mM d GTP, 2 µl 0.25 mM d TTP, 5 µl 10 x nick buffer (500 mM Tris-HCl buffer, 50 mM MgCl, 100 mM β-mercapto-ethanol, 500 µg/ml Bovine serum albumin), 2 µl 0.5 M NaCl, 25 µl *A. orana* or *B. brassicae* NPV-DNA at a concentration of 20 µg/ml, 8 µl water,

5 µl DNase 10^{-8} g/ml, 1 µl DNA Polymerase I. After nick-translation the reaction mixture was extracted once with phenol and once with a mixture of chloroform--isoamyl alcohol (24 : 1). After these extractions, the mixture was passed over a G-50 Sephadex column to separate the DNA from the remaining substrates.

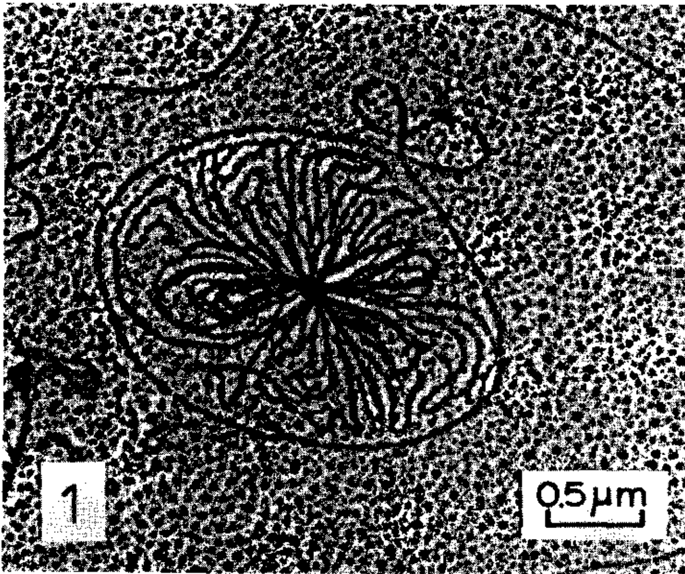
Reassociation kinetics. Reassociation was based on the method of Sharp et al. (1974). Before annealing, the labelled and unlabelled DNAs were mixed and degraded to nucleotide segments 200 to 300 bases in length by boiling in 0.3 N NaOH for 20 min. The solutions (500 µl) were immediately immersed in ice. After cooling, the solution was adjusted to *p*H 6.8 with ice cold 140 µl phosphate buffer (1 M), 40 µl SDS (10%), 140 µl NaCl (5 M), 158.4 µl water and 21.6 µl HCl (6 N). DNA was renatured by heating fragmented DNA in a closed ampule to 68°C. Renaturation was stopped by dilution of the samples with 1 ml of ice cold 0.14 M sodium phosphate buffer, *p*H 6.8. The extent of renaturation was determined on hydroxylapatite columns at 60°C. Single-stranded DNA was eluted with 0.14 M sodium phosphate buffer and double-stranded DNA with 0.4 M sodium phosphate buffer.

DNA analysis by restriction endonuclease Eco RI. Restriction enzyme analysis with *Eco* RI was performed according to Sussenbach and Kuijk (1977). DNA was digested in 0.09 M Tris-HCl buffer plus 0.01 M $MgCl_2$ (*p*H 7.9). The reaction was stopped by addition of sodium acetate to 0.2 M and 1 vol of chloroform-isoamyl alcohol (24 : 1). After extraction and low-speed centrifugation, the aqueous phase was mixed with 3 vol of ethanol and DNA was precipitated by centrifugation. The precipitated DNA was dissolved in 20 mM Tris-HCl buffer, 1 mM EDTA (*p*H 7.5) and analysed by electrophoresis in 1.4% agarose gels. Agarose (Bio-Rad, Richmond, California) was dissolved in E buffer containing 0.04 M Tris-HCl buffer, 0.05 M sodium acetate, 0.001 M EDTA (*p*H 7.8), and 0.5 µg/ml of ethium bromide. Electrophoresis was at room temperature at 2 to 3 V/cm.

RESULTS

Electron microscopy of NPV-DNAs

When DNA obtained by lysis of virus with SDS, was dialysed against 0.02 M Tris-HCl buffer plus 0.03 M EDTA (*p*H 8.5) and then analysed by electron microscopy, it was observed that about 65% of the DNA molecules had the typical structures of supercoiled forms with some additional foldings (Fig. 1 and 2). When the DNA was purified by modification of the method of Gafford and Randal (1967) and analysed by electron microscopy, these typical forms were not found. Instead three



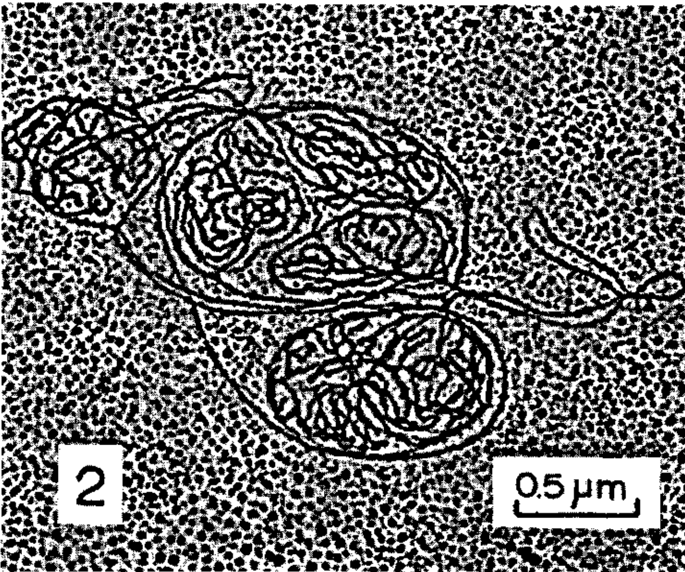


Fig. 1 and 2. The *A. orana* (1) and the *B. brassicae* NPV-DNA (2) in the supercoiled form with some additional foldings. Virus was lysed with 2% SDS at 60° for 30 min. Released DNA was dialysed against 0.02 M Tris-HCl buffer plus 0.03 M EDTA (*p*H 8.5) and then spread on water hypophase with 20% formamide.

different forms of DNA molecules were seen in the following proportions: about 40% highly twisted circles, about 50% relaxed circles and in most preparations less than 10% linear molecules of various length. Figures 3 and 4 show different forms of the *A. orana* NPV-DNA. The same forms were also observed for the *B. brassicae* NPV-DNA.

Determination of molecular weights of NPV-DNA by electron microscopy

The Kleinschmidt technique was used in this study to determine the molecular weights of NPV-DNA of *A. orana* and of *B. brassicae*. To obtain reproducible results, only the relaxed circles on the same grid with marker DNA were used for length determinations. The distribution of size classes of the *A. orana* NPV-DNA in Fig. 5 shows a peak at 34 μm and a small peak at about 130 μm. The largest molecule observed was almost 5 times larger than the fundamental unit. The distribution of size classes of the *B. brassicae* NPV-DNA circles in Fig. 6 shows a peak at 45 μm and peaks at 130 and 180 μm. The largest molecule observed was 4 times the fundamental unit.

The molecular weights (M) were calculated from the formula used by Lang (1970), $M = M'L$, where M' means Daltons per μm and L is the length of DNA molecule in μm. M' calculated in our study by calibration of bacteriophage PM2 was 1.98×10^6 Daltons per μm. With this formula, the molecular weights of the NPV-DNA of *A. orana* and of *B. brassicae* are 6.7×10^7 and 8.9×10^7 Daltons, respectively. The marker DNA was co-spread and photographed with NPV-DNAs.

Reassociation kinetics analysis of the NPV-DNA of A. orana and of B. brassicae

To check the molecular weights established by electron microscopy, these were also determined by reassociation kinetics analysis. Denatured DNA from NPV of *A. orana* and of *B. brassicae* was reannealed at known DNA concentrations and the rate of reassociation was determined employing hydroxylapatite chromatography. Adenovirus Type 5 DNA (23×10^6 Daltons, Philipson and Lindberg, 1974) was used as a reference DNA. Reassociation analysis was performed as described in Materials and Methods. As shown in Table 1 and Fig. 7, DNA of both viruses reassociate with kinetics approaching ideal second order kinetics. In neither preparation was a rapidly reannealing fraction detected. Thus, the genomes do not contain repetitive sequences. The genome sizes (Table 2) were derived from the linear relationship between the $Cot_{\frac{1}{2}}$ values of NPV-DNA and the $Cot_{\frac{1}{2}}$ values of Adenovirus Type 5 DNA

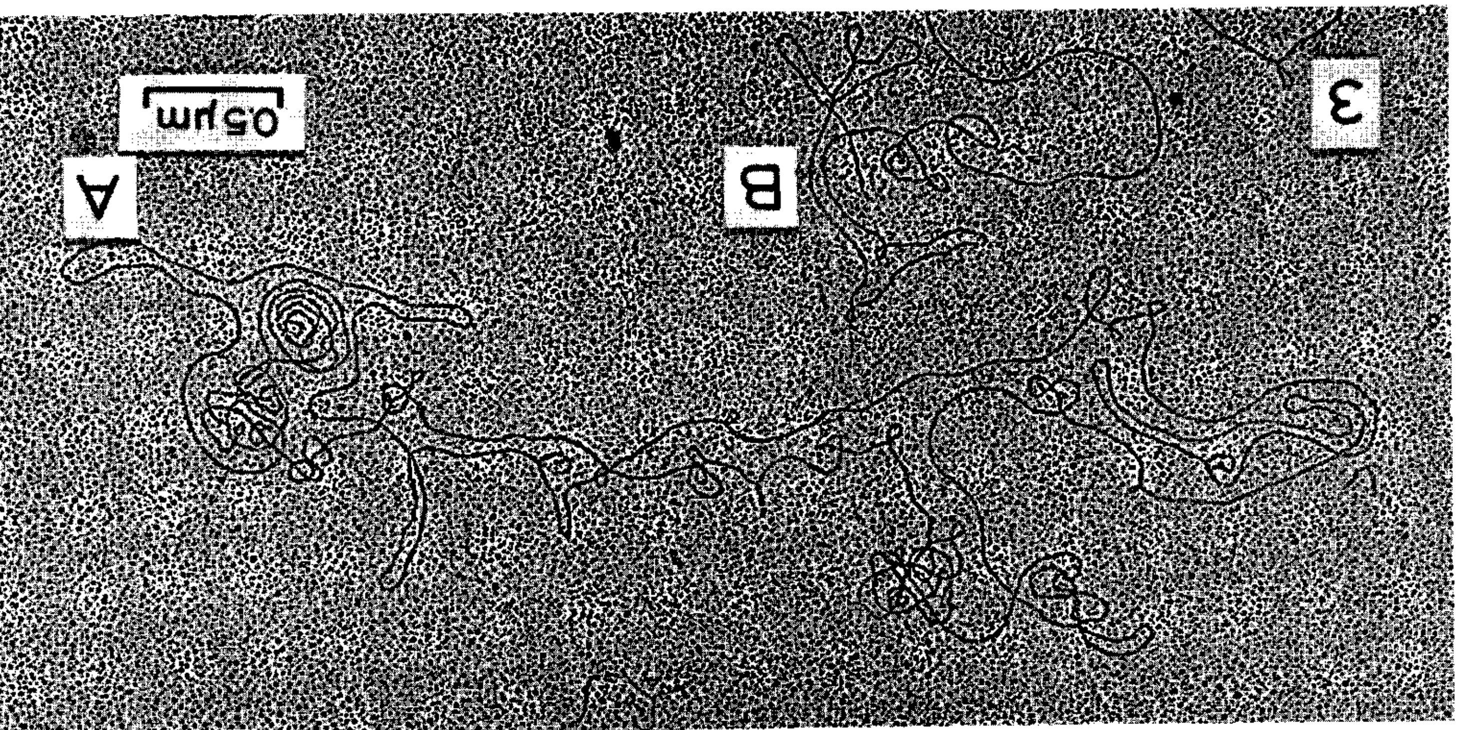
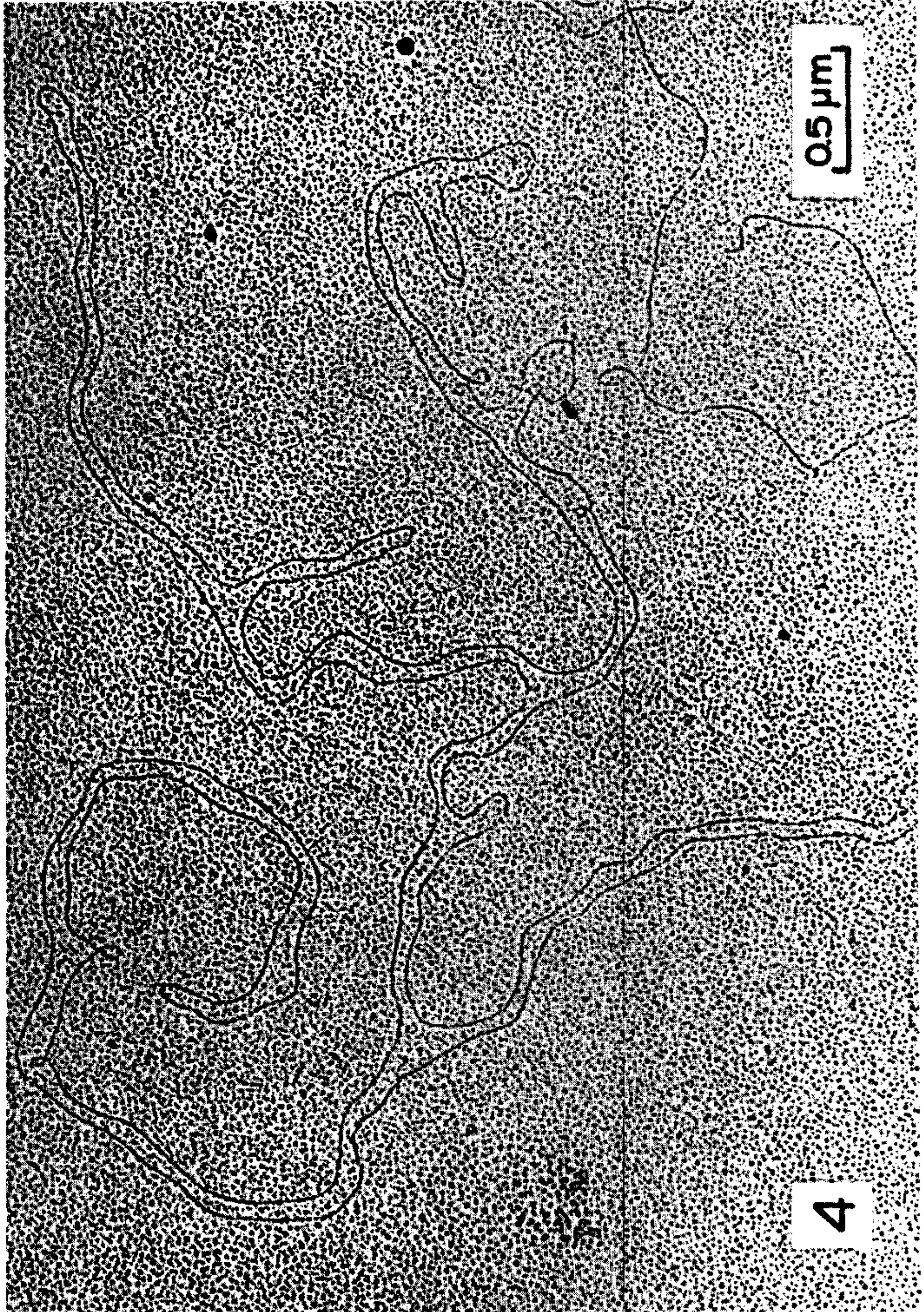


Fig. 3 and 4. Different forms of the NPV-DNA of *A. orana*. A supercoiled DNA molecule (A). A linear molecule with free end (B) and a relaxed circular DNA molecule (C). DNA was purified by modification of the method of Gafford and Randal (1967) and spread on water hypophase with 20% formamide.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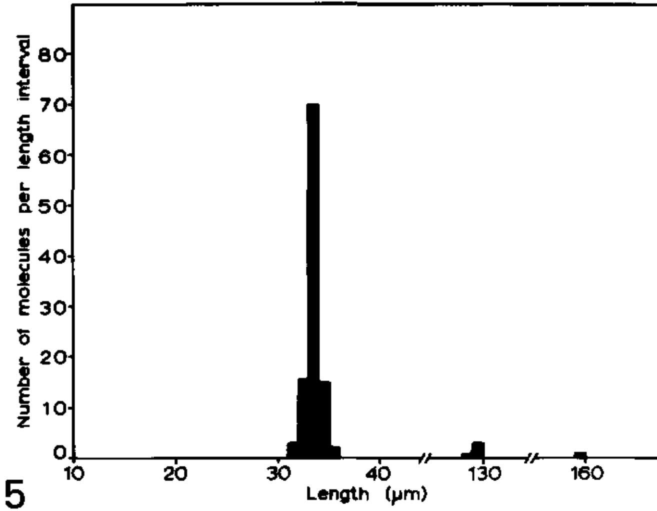

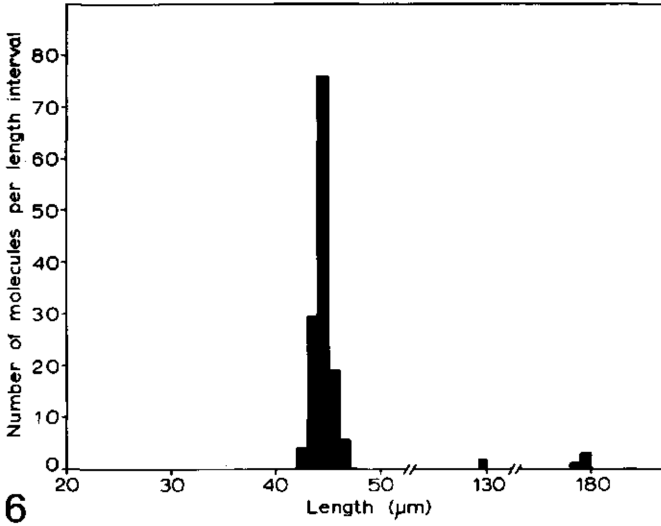

Fig. 5 and 6. Histograms of the length distribution of the NPV-DNA of *A. orana* and of *B. brassicae*. A total number molecules 112 and 139, of the *A. orana* and of the *B. brassicae* NPV-DNA respectively, were examined. DNA of bacteriophage PM2 was used as an internal standard. Length intervals are 1 μm.

Table 1. **Reassociation kinetics of NPV-DNA of *A. orana* and of *B. brassicae*, respectively, and Adenovirus Type 5 DNA**

Time of sampling in hr	^{32}P-lab. *A.orana* NPV-DNA & homologous unlab. fragments		^{32}P-lab. *B.brassicae* NPV-DNA & homologous unlab. fragments		^{32}P-lab. Adenovirus 5-DNA & homologous unlab. fragments	
	cpm in fss	$^{1}/$fss	cpm in fss	$^{1}/$fss	cpm in fss	$^{1}/$fss
0.0	1,400	1.000	2,200	1.000	1,950	1.000
0.1	1,388	1.008	2,184	1.007	1,852	1.052
0.2	1,370	1.021	2,164	1.016	1,774	1.110
0.3	1,355	1.037	2,151	1.022	1,696	1.150
0.4	1,330	1.050	2,112	1.040	1,593	1.200
0.5	1,302	1.070	2,101	1.045	1,528	1.275
1.0	1,172	1.195	2,000	1.100	1,316	1.480
1.5	1,102	1.270	1,833	1.200	1,146	1.700
2.0	1,077	1.300	1,760	1.250	1,010	1.926
2.5	-	-	-	-	907	2.150
3.0	952	1.470	1,599	1.375	-	-
6.0	721	1.941	1,256	1.750	-	-
8.0	622	2.252	1,102	1.996	-	-

The reassociation mixture of *A. orana* NPV-DNA contained 1.9×10^{-3} μg of ^{32}P-labelled and 0.6 μg of unlabelled fragments. The mixture of *B. brassicae* contained 2.0×10^{-3} μg of ^{32}P-labelled and 0.6 μg of unlabelled fragments and the mixture of Adenovirus Type 5 DNA (marker DNA) consisted of 1.5×10^{-3} μg of ^{32}P-labelled and 0.6 μg unlabelled fragments. All reaction mixtures had a volume of 1 ml. For the analysis of the *A. orana* and of the *B. brassicae* two samples of 1 ml were used.

cpm = counts per min, $^{1}/$fss = $^{1}/$single-stranded fraction.

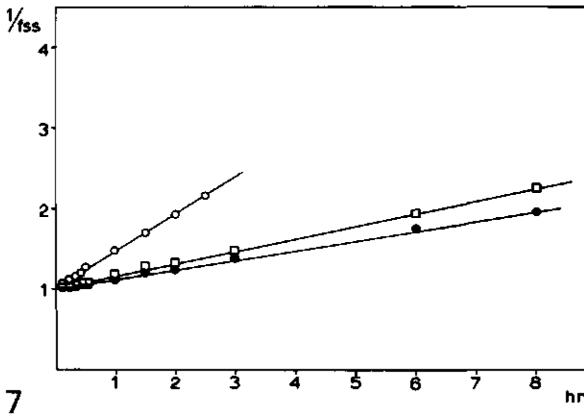

Fig. 7. Reassociation of the *A. orana* and of the *B. brassicae* NPV-DNA, respectively. Reassociation of fragments of *A. orana* (□—□). The reassociation mixture contained 1.9×10^{-3} µg (1,400 cpm) of ^{32}P-labelled *A. orana* NPV-DNA and 0.6 µg of unlabelled homologous fragments. Reassociation of *B. brassicae* NPV-DNA (●—●). The reassociation mixture contained 2.0×10^{-3} µg (2,200 cpm) of ^{32}P-labelled NPV-DNA of *B. brassicae* and 0.6 µg of unlabelled homologous fragments. Reassociation of Adenovirus Type 5 DNA (marker DNA) (○—○). The mixture contained 1.5×10^{-3} µg (1,950 cpm) of ^{32}P-labelled Adenovirus Type 5 DNA and 0.6 µg of unlabelled homologous fragments. All samples had a volume of 1 ml. The single-stranded fraction (fss) was determined by hydroxylapatite chromatography.

Table 2. The molecular weights of *A. orana* and of *B. brassicae* NPV-DNA

DNA of	$Cot_{\frac{1}{2}}$	Equivalent molecular weight ★
A. orana NPV	$3.8 \times 10^{-2} \pm 0.0020$	6.6×10^{7}
B. brassicae NPV	$4.9 \times 10^{-2} \pm 0.0022$	8.6×10^{7}
Adenovirus Type 5	$1.3 \times 10^{-2} \pm 0.0009$	2.3×10^{7}

★ The molecular weights of *A. orana* and *B. brassicae* NPV-DNA were calculated assuming a linear relationship between the $Cot_{\frac{1}{2}}$ values and genome sizes. The molecular weight of Adenovirus 5 DNA was taken as a standard (2.3×10^{7}, Philipson and Lindberg, 1974). Standard deviations of *A. orana* and *B. brassicae* NPV-DNA are calculated from 6 determinations and of Adenovirus Type 5 DNA from 3 determinations.

which was used as marker (Sharp et al., 1974). It appears that *A. orana* NPV-DNA is about 2.9 times more complex than Adenovirus Type 5 DNA and that the *B. brassicae* NPV-DNA is about 3.75 times more complex. Since Adenovirus Type 5 DNA

has a molecular weight of 23 x 10^6 Daltons, it can be calculated that the molecular weight of *A. orana* NPV-DNA is 6.6 x 10^7 and the molecular weight of *B. brassicae* NPV-DNA is 8.6 x 10^7 Daltons. The molecular weights obtained by reassociation kinetics show a very good agreement with those obtained by electron microscopy. The results also indicate that the genomes contain only unique sequences.

Buoyant density analysis

The buoyant density of the NPV-DNA of *A. orana* and of *B. brassicae* was determined by ultracentrifugation in CsCl. As a density marker DNA, DNA of *E. coli* (ρ = 1.710 g/cm^3, Szybalski, 1968) and DNA of bacteriophage T4 (ρ = 1.694 g/cm^3) was used (Fig. 8). The relative buoyant density of *A. orana* NPV-DNA,

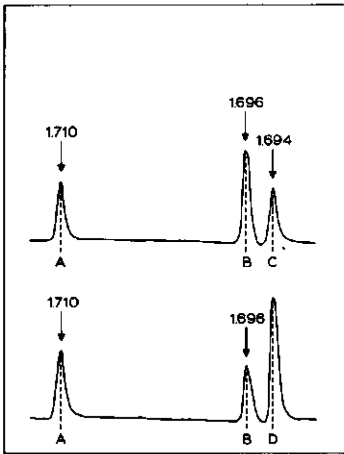

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Fig. 8. Analytical buoyant-density analysis of: the *B. brassicae* NPV-DNA (B). The *E. coli* DNA (A) and the bacteriophage T4 DNA (C) were used as markers (Top figure). The *A. orana* NPV-DNA (D). The *E. coli* DNA (A) and the *B. brassicae* NPV-DNA were used as markers (Bottom figure).

1.694 g/cm^3 and of *B. brassicae* NPV-DNA 1.696 g/cm^3 was calculated by the equation of Mandel et al. (1968). The corresponding (G+C) content of *A. orana* NPV-DNA 34% and of *B. brassicae* NPV-DNA 36.7% were obtained from the linear relation of Schildkraut et al. (1962).

Thermal denaturation profiles

Melting profiles were made for the NPV-DNA of *A. orana* and of *B. brassicae* with DNA of T4 bacteriophage as standard [(G+C) content 34%, Wyatt and Cohen, 1953]. Fig. 9 shows that the approximate melting point (Tm) for the NPV-DNA of *A. orana* and of *B. brassicae* is 83.4^{o}C and 84.4^{o}C, respectively and for the

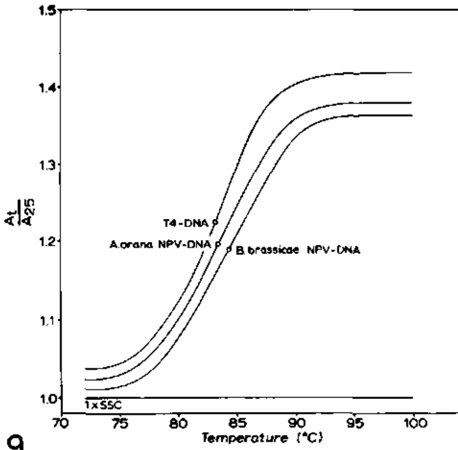

Fig. 9. Thermal melting profiles (Tm) of the nuclear polyhedrosis DNA of *A. orana* and of *B. brassicae*. Measurements were made in 1 x SSC in a Gilford Model 2400 recording spectrophotometer. Tm values were calculated from the formula of Mandel and Marmur (1968), using T4 bacteriophage DNA (34% (G+C) content, Wyatt and Cohen, 1953) as standard. The DNA preparations did not contain covalently closed DNA; they were sheared by Vortex, 2 times for 15 sec. The experiment was repeated four times.

DNA of T4 bacteriophage 83.2^{0}C. The Tm values correspond to a (G+C) content of 34.5 for *A. orana* NPV-DNA and 37% for *B. brassicae* NPV-DNA when calculated from the formula of Mandel and Marmur (1968). The values are in good agreement with those obtained from buoyant density analysis. The hyperchromicity of NPV-DNAs was about 37-39%.

Sequence homology between A. orana and B. brassicae NPV-DNA

The extent of sequence homology between the DNA of these two viruses was determined by reannealing of ^{32}P-labelled *A. orana* NPV-DNA fragments in the presence of *B. brassicae* unlabelled NPV-DNA fragments (0.9, 1.8 or 2.7 µg/ml, respectively). As a positive control, ^{32}P-labelled *A. orana* NPV-DNA was reannealed with unlabelled homologous fragments (0.9, 1.8 or 2.7 µg/ml, respectively) while as negative control a mixture of ^{32}P-labelled *A. orana* NPV-DNA and unlabelled *E. coli* DNA fragments (0.9, 1.8 or 2.7 µg/ml, respectively) was used. The value of $Cot_{\frac{1}{2}}$ of *A. orana* NPV-DNA, which reannealed in the presence of *E. coli* DNA fragments, was the same as that of *A. orana* NPV-DNA, which reannealed in the presence of *B. brassicae* NPV-DNA (3.8×10^{-2}). The rate of reassociation of *A. orana* NPV-DNA, which reannealed in the presence of *B. brassicae* NPV-DNA, did not increase when the concentration of *B. brassicae* NPV-DNA fragments was increased. The data are given in Table 3 and Fig. 10. There is no sequence homology between *A. orana* and *B. brassicae* NPV-DNA.

Table 3. **Reassociation kinetics of the *A. orana* NPV-DNA in the presence of *B. brassicae* NPV-DNA**

Time of sampling in hr	^{32}P-lab. *A. orana* NPV-DNA & unlab. *B. brassicae* NPV-DNA		^{32}P-lab. *A. orana* NPV-DNA & unlab. *A. orana* NPV-DNA		^{32}P-lab. *A. orana* NPV-DNA & unlab. *E. coli* DNA	
	cpm in fss	$^{1}/fss$	cpm in fss	$^{1}/fss$	cpm in fss	$^{1}/fss$
0	1,529	1.000	1,507	1.000	1,537	1.000
1	1,525	1.000	1,200	1.250	1,536	1.000
2	1,527	1.000	1,017	1.474	1,536	1.000
3	1,518	1.001	856	1.751	1,528	1.001
4	1,518	1.001	769	1.950	1,526	1.002
6	1,515	1.002	624	2.403	1,526	1.002
8	1,516	1.002	-	-	1,515	1.010
54	1,497	1.015	-	-	1,508	1.014
78	1,494	1.017	-	-	1,505	1.016

The competition reaction mixture contained 2.06×10^{-3} µg of ^{32}P-labelled *A. orana* NPV-DNA and 0.9 µg of unlabelled *B. brassicae* NPV-DNA. The positive control contained 2.03×10^{-3} µg of ^{32}P-labelled *A. orana* NPV-DNA and 0.54 µg of unlabelled homologous fragments. The negative control contained 2.07×10^{-3} µg of ^{32}P-labelled *A. orana* NPV-DNA and 0.9 µg of unlabelled *E. coli* DNA fragments.

cpm = counts per min, $^{1}/fss$ = $^{1}/$single-stranded fraction.

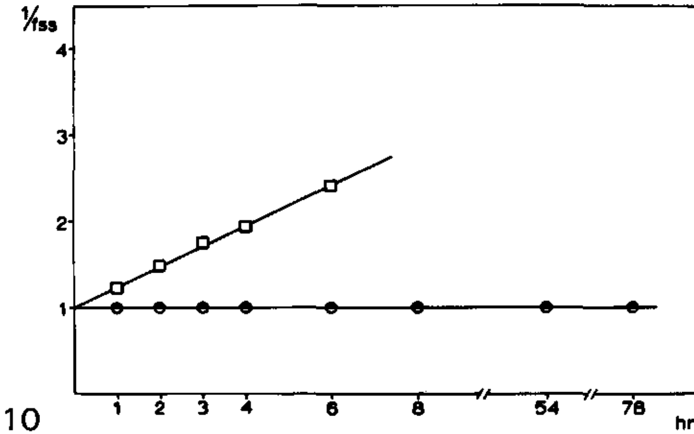

Fig. 10. Reassociation of *A. orana* NPV-DNA in the presence of *B. brassicae* NPV-DNA (●—●). The reaction mixture contained 2.06 x 10^{-3} μg (1,529 cpm) of ^{32}P-labelled *A. orana* NPV-DNA and 0.9 μg of *B. brassicae* unlabelled NPV-DNA. Positive control (□—□). The reaction mixture contained 2.03 x 10^{-3} μg (1,507 cpm) of ^{32}P-labelled *A. orana* NPV-DNA and 0.54 μg of homologous unlabelled fragments. Negative control (○—○). The reaction mixture contained 2.07 x 10^{-3} μg (1,537 cpm) of ^{32}P-labelled *A. orana* NPV-DNA in the presence of 0.9 μg of unlabelled *E. coli* DNA. All reaction mixtures had a volume of 1 ml.

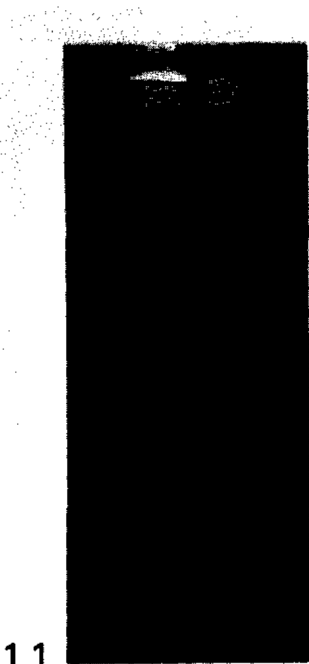

Fig. 11. Patterns of DNAs cleaved by restriction endonuclease *Eco* RI in 1.4% agarose gels. The *Adoxophyes orana* NPV-DNA (1). The *Barathra brassicae* NPV-DNA (2). Digestion and electrophoresis are described in Materials and Methods.

Comparison of the A. orana and of the B. brassicae NPV-DNAs by cleavage with restriction endonuclease Eco RI enzyme

In addition to the comparison of the two DNAs by competition hybridization the *A. orana* and the *B. brassicae* NPV-DNA were also digested by *Eco* RI enzyme. As shown in Figure 11, the NPV-DNA of *A. orana* and of *B. brassicae* give completely different patterns when the digestion products were fractionated on 1.4% agarose gels. Both DNAs are cleaved into at least 13 fragments but no fragments of corresponding electrophoretic mobility are observed.

DISCUSSION

In the past, the NPV of *A. orana* and of *B. brassicae* were identified by the shape of the polyhedral bodies and by the morphology of their viruses (Ponsen et al., 1965; Ponsen and De Jong, 1964; Ponsen and Bruinvis, 1963). However, the differences in shape and morphology do not seem to be uniquely associated with a particular biological characteristic of different NPVs (Shigematsu and Suzuki, 1971). To elucidate further the relationship between these viruses, we determined some physical properties of their DNAs.

As shown by electron microscopy and thermal denaturation nucleic acid material isolated from NPV of *A. orana* and of *B. brassicae* larvae (resistent to ribonuclease A - Type XI) consists of circular double-stranded DNA. Electron microscopy of DNA released from nucleocapsids further revealed that the NPV-DNAs were obtained in highly supertwisted relaxed circular and double-stranded linear structures. The observations of different forms of NPV-DNAs agree with the results of caesium chloride-analysis reported by Summers and Anderson (1973), and Harrap et al. (1977) for *Spodoptera* sp. NPV-DNAs and Summers and Anderson (1972b) for granulosis virus of *Trichoplusia ni* and *Spodoptera frugiperda*. They are also consistent with electron microscopic observations of Bud and Kelly (1977) for *Spodoptera* sp. NPV-DNAs and of Brown et al. (1977) for granulosis virus of *Pieris brassicae*.

From experience gained during this work and according to Bud and Kelly (1977), we think that the environmental conditions of the DNA outside the virus particle induce either supercoiling or relaxation of the DNA molecule. On the other hand, the observation of supercoiled molecules in DNA preparations obtained by the shortened purification method suggests that the molecules may be packed in the virus in the supercoiled form with additional foldings. The formation of a

supercoil is probably the first step in fitting the large DNA molecules into the small nucleocapsid.

To distinguish the DNAs from the NPV of *A. orana* and of *B. brassicae* their lengths were studied by electron microscopy. These two DNAs differ in length but they are both large molecules similar to those observed for granulosis or other NPV-DNAs (Kok et al., 1972; Burgess, 1977; Rohrmann and Beaudreau, 1977; Scharnhorst et al., 1977; Tweeten et al., 1977). Electron microscopy has also shown that DNA of the NPV of *A. orana* and of *B. brassicae* are homologous in size, in contrast to the DNA molecules isolated from some other NPVs which were found to be heterogenous in size (Kok et al., 1972; Scharnhorst et al., 1977). However, a small fraction of DNA molecules of both viruses were oligomers. The genome sizes of DNA of these viruses were also determined by reassociation kinetics. A rapidly annealing fraction was not detected, indicating that within the range of detection of the hydroxylapatite columns analysis, no repetitive sequences are present. Kelly (1977) reported about 2-3% of intragenome homology for the NPV-DNAs of *Spodoptera* sp. However this amount can be indicated as the range of detection for this method. The molecular weights obtained by reassociation kinetics analysis of the NPV-DNA of *A. orana* and of *B. brassicae* are in agreement with the values obtained from the length measurements.

To elucidate further the relationship between these NPV-DNAs, we performed competition hybridization to determine the nucleotide sequence homology between both DNAs using ^{32}P-labelled *A. orana* NPV-DNA and increasing amounts of unlabelled *B. brassicae* NPV-DNA (0.9 to 2.7 μg/ml). In contrast to Kelly (1977), who found sequence homology between four NPV-DNAs of *Spodoptera* sp., we found no competition between the NPV-DNAs of the *A. orana* and of the *B. brassicae*, indicating that no homology exists between both DNAs.

As for NPV-DNAs of *Orgyia pseudotsugata* (Rohrmann et al., 1978), also for NPV-DNA of *A. orana* and of *B. brassicae* completely different *Eco* RI cleavage patterns were obtained. However the comparison of these NPV-DNAs by *Eco* RI cleavage patterns is less sensitive than the comparison by reassociation kinetics. The only purpose of the gel patterns of restricted DNA is to make our viruses comparable with the viruses employed in other laboratories.

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IV SOME PROPERTIES OF THE GENOME OF *ADOXOPHYES ORANA* (LEPIDOPTERA: TORTRICIDAE) AND OF *BARATHRA BRASSICAE* (LEPIDOPTERA: NOCTUIDAE)

SUMMARY

The host DNA was isolated from the 4th instar larvae of *Adoxophyes orana* and from that of *Barathra brassicae*. The haploid genome size of *A. orana* 4.2×10^{10} and that of *B. brassicae* 8.4×10^{10} Daltons, were determined from reassociation data based upon hyperchromicity. Reassociation kinetics also imply that about 90% of the *A. orana* and 91% of the *B. brassicae* genome nucleotide sequences are unique sequences (single-copy). The (G+C) content, estimated by thermal denaturation, was 36.2% for the *A. orana* genome and 35.8% for the *B. brassicae* genome.

INTRODUCTION

Hybridization of nuclear polyhedrosis virus (NPV) DNA of *A. orana* and of *B. brassicae* with host genome of homologous insects revealed the presence of viral sequences in host DNA samples. However, it was impossible to calculate the amount of viral sequences per diploid quantity of host DNA or to study further viral genome because the host genome of both insects had not been characterized molecularly.

The present report describes the initial characterization of host genome of *A. orana* and of *B. brassicae* larvae. It includes: genome size, estimation of per cent repeated sequences in the genome and the (G+C) content.

MATERIALS AND METHODS

Insects. The eggs of *A. orana* were obtained from the Laboratory of Entomology, Agricultural University, Wageningen. The *B. brassicae* cultures supposedly having a latent NPV were obtained from Dr. L. Varjas, Research Institute for Plant Protection, Budapest.

The *A. orana* larvae were reared solitarily in tubes on the diet developed by Adkisson et al. (1960). The *B. brassicae* larvae were mass-reared in preserving jars on an artificial diet developed by Nagy (1970). Both insects were reared at $20^{o}C$, a relative humidity of 70% and 12 hr of light per day.

Isolation of host DNA. The procedure used is a combination and modification of the methods of Gall and Atherton (1974) and Gross-Bellard et al. (1973). Five g of fourth instar larvae, reared from surface sterilized eggs, were ground at $4^{o}C$ and then solution of 0.05 M Tris-HCl, 0.025 M KCl, 0.005 M magnesium acetate and 0.35 M sucrose (*p*H 7.6) was added. The filtered suspension was centrifuged at 5,900*g* for 10 min. The pellet was suspended in 0.05 M Tris-HCl buffer, 0.1 M EDTA, 0.5% β-mercaptoethanol, 0.5% sodium lauryl sulphate (SLS) and 300 μg/ml Proteinase K (*p*H 7.7). After incubation for 90 min at $37^{o}C$, SLS was added to a concentration of 2% and the solution was incubated at $60^{o}C$ for 30 min. The viscous solution was stirred with an equal volume of water-saturated phenol for 30 min. The phenol treatment was repeated twice followed by centrifugation at 12,000*g* for 30 min. The aqueous phase containing DNA was dialysed against 1 x SSC plus 10 mM EDTA (*p*H 7.4). The crude DNA was treated with pancreatic RNase 150 μg/ml, RNase T_1 495 units/ml and α-Amylase 375 μg/ml for 2 hr at $37^{o}C$. Then Pronase 300 μg/ml was added and the incubation at $37^{o}C$ was continued for 2 hr to reduce the protein content of the solution. The digestion was followed

by dialysing the DNA against 0,02 M Tris-HCl buffer plus 10 mM EDTA (*p*H 8.5). Purification was accomplished by isopycnic centrifugation in CsCl. The solution was centrifuged in SW-41 rotor tubes at 30,000 rev/min for 36 hr at 25°C.

Thermal denaturation. Thermal denaturation curves (Tm) were made in 1 x SSC (0.015 M sodium citrate and 0.15 M NaCl), *p*H 7.0 according to Mandel and Marmur (1968) in a Gilford Model 2400 spectrophotometer. The DNA samples (sheared by Vortex 2 times for 15 sec) at a final concentration of 50 μg/ml were dialysed against 1 x SSC, at 4°C, three changes of 16 - 18 hr each. During melting, the temperature was raised by 0.1^{o}C per min and the absorbance was recorded every minute. The (G+C) content was calculated from Tm values according to Marmur and Doty (1961).

Reassociation of DNA. Sheared DNA used for reassociation experiments was prepared by passing the DNA, suspended in 2 x SSC (0.030 M sodium citrate and 0.30 M NaCl), *p*H 7.0, twice through a French pressure cell at 20,000 lb/in^2 and a rate of 0.8 - 2 drops per sec (Kelly and Avery, 1974). Nearly all DNA fragments of the same proportion were within the range 350 to 700 nucleotide pairs. One typical distribution example is given in Fig. 1.

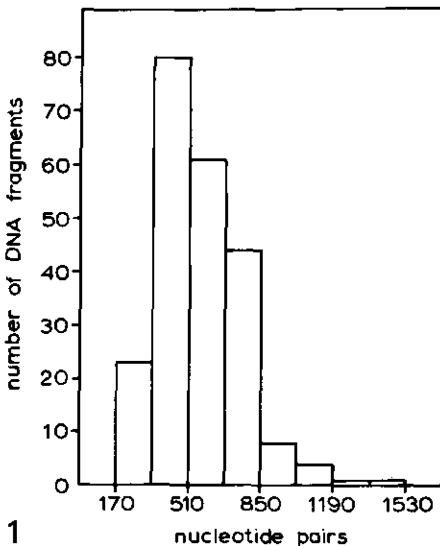

Fig. 1. Size distribution of the *E. coli* DNA fragments. DNA resuspended in 2 x SSC was passed twice through a French pressure cell at 20,000 lb/in^2 and a rate of 0.8 - 2 drops per sec. Data presented were obtained by measurements of DNA fragments spread by Kleinschmidt technique. (Kleinschmidt, 1968; Davis et al., 1971). The number of nucleotide pairs was calculated with the formula of Britten and Kohne (1968).

The sheared DNA was heat denatured by immersing the samples for 10 min in a waterbath at 100°C. The solutions were quickly poured into the preheated cuvettes in the cuvettes-holder compartment of a Gilford Model 2400 spectrophotometer. The suspensions were rapidly cooled to the optimum renaturation temperature of about 25°C below the Tm value (Marmur and Doty, 1961). A guanine solution absorbance of about 1.0 was always included as a blank and to allow for correction for any electronic drift. As marker DNA, DNA of *E. coli* [2.7 x 10^{9} Daltons, Cairns (1963)] was used. The recorder was switched on simultaneously. The reassociation was followed for 14 hrs.

RESULTS

Thermal denaturation profiles

Melting profiles were made for the host DNA of *A. orana* and of *B. brassicae*, using the DNA of *E. coli* as standard [(G+C) content 51.0%, Marmur and Doty (1962)]. As seen in Fig. 2, the approximate melting points for the host DNA of

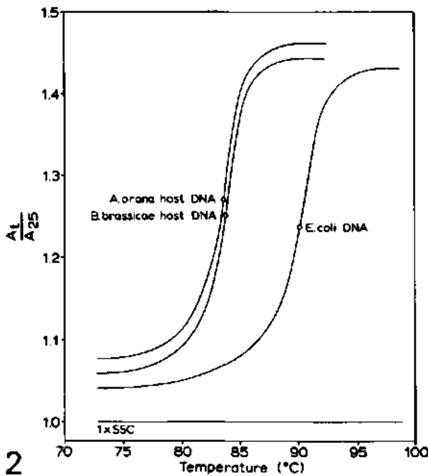

Fig. 2. Thermal melting profiles (Tm) of the host DNA of *A. orana* and of *B. brassicae*. As marker DNA, DNA of *E. coli* [51% (G+C) content, Marmur and Doty (1962)] was used. DNAs were suspended in 1 x SSC and measurements were carried out in a Gilford Model 2400 recording spectrophotometer. The values were calculated from the formula of Mandel and Marmur (1968). The DNA preparations were sheared by vortexing twice for 15 sec. The experiment was repeated four times.

A. orana and of *B. brassicae* were 84.1 and 84.0^{O}C, respectively and for the DNA of *E. coli* it was about 90.2^{O}C. The melting points correspond to a (G+C) content of 36.2% for the host DNA of *A. orana* and 35.8% for the host DNA of *B. brassicae* as calculated with the formula of Mandel and Marmur (1968). Hyperchromicity of DNA was about 37 - 39%.

Kinetic complexity of the A. orana and the B. brassicae genome

Sheared DNA samples (of about 520 nucleotide pairs) of the *A. orana* host DNA (98 μg/ml) and *E. coli* DNA (48 μg/ml) which was run simultaneously as a size standard were heat denatured and allowed to reassociate in 2 x SSC at 71.6°C. The hyperchromicity was measured in order to follow the rate of hybrid formation.

The second-order renaturation rate constant K_2 was calculated from the equation of Wetmur and Davidson (1968)

$$\frac{A_o - A}{A_t - A} = \frac{K_2 \times P_T}{2} \times t + 1$$

where: A is the absorbance of native DNA at 260 nm, A_o is the absorbance of denatured DNA, A_t is the absorbance of partially reassociated DNA (at reassociation temperature) at time t, P_T is the total DNA phosphate concentration. P_T was taken as $1.47 \times 10^{-4} \times A$ mole 1^{-1}. For *A. orana* and for *E. coli* in this experiment, K_2 of DNA was 0.0867 and 1.349 mole^{-1}sec^{-1}1, respectively. The second--order rate plot for the data of the *A. orana* host DNA is seen in Fig. 3. Because

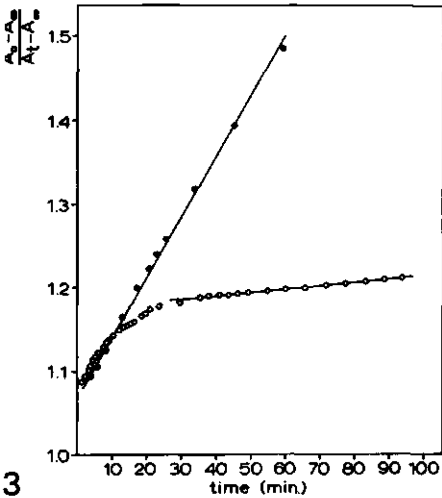

Fig. 3. Second-order rate plot for the reassociation data of the *A. orana* genome. The *A. orana* DNA (○—○) (98 μg/ml) and the *E. coli* DNA (●—●) (48 μg/ml) were reassociated at 71.6°C in 2 x SSC. The slope of the *A. orana* genome plot is 0.125×10^{-4} sec^{-1}. The K_2 is 0.0867 mole^{-1} sec^{-1} 1. The slope of the *E. coli* DNA plot is 0.952×10^{-4} sec^{-1}. The K_2 is 1.349 mole^{-1} sec^{-1} 1.

both DNA were sheared to fragments of the same length, there was no correction applied for the calculation of the K_2 value.

The kinetic complexity of the *A. orana* genome was estimated from the relation:

$$\frac{K_2 \text{ of } E.\ coli}{K_2 \text{ of } A.\ orana} = \frac{N_d \text{ of } A.\ orana}{N_d \text{ of } E.\ coli}$$

where K_2 is the second-order renaturation rate constant, and N_d is the kinetic complexity of genome. N_d for *E. coli* DNA is 2.7×10^9 Daltons (Cairns, 1963). The calculation showed that the kinetic complexity of *A. orana* genome was about 4.2×10^{10} Daltons. From Fig. 3 it is seen that a small fraction of DNA reassociated fast. The fast fraction of about 10% was estimated from the extrapolation of the amount of slow fraction to its zero time (Wells and Birnstiel, 1969).

The kinetic complexity of the *B. brassicae* genome was calculated in the same way as for *A. orana*. The *B. brassicae* DNA (93.0 µg/ml) and the marker DNA, DNA of *E. coli* (47.1 µg/ml) were sheared to about 520 nucleotide pairs, heat denatured and allowed to reassociate in 2 x SSC at 70.5°C. The second-order reassociation rate constant K_2 was 0.05409 and 1.683 $mole^{-1} sec^{-1}$ 1 for the host DNA of *B. brassicae* and *E. coli* DNA, respectively. The second-order rate plot for the *B. brassicae* genome is seen in Fig. 4. The *E. coli* DNA data follow the second-order

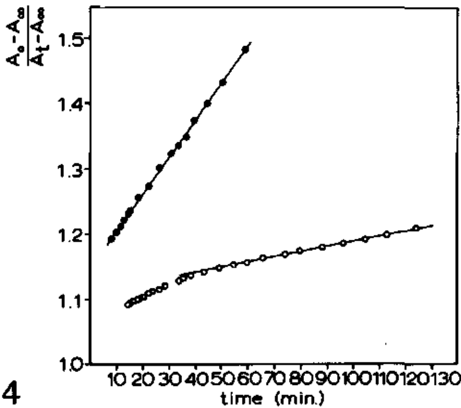

Fig. 4. Second-order rate plot for the data of the *B. brassicae* genome. The *B. brassicae* DNA (○—○), concentration of 93 µg/ml and the *E. coli* DNA (●—●), concentration of 47.1 µg/ml were reassociated at a temperature of 70.5°C in 2 x SSC. The slope of the *B. brassicae* DNA plot is 0.73955×10^{-5} sec^{-1}, the K_2 value is 0.0541 $mole^{-1}$ sec^{-1} 1. The slope of the *E. coli* DNA plot is 0.1165×10^{-3} sec^{-1}, the K_2 value is 1.683 $mole^{-1}$ sec^{-1} 1.

rate curve. The data of *B. brassicae* showed that a small fraction of about 9% reassociate fast. As for *A. orana* this fraction was estimated from the extrapolation of the amount of slow fraction to its zero time. The kinetic complexity for *B. brassicae* was calculated to be 8.4×10^{10} Daltons. The kinetic complexity estimated in this manner correspond to the minimum amount of DNA in which each nucleotide sequence is represented once. The amount of DNA per cell will correspond to this estimate when the cell is haploid in genetic sense.

DISCUSSION

The (G+C) content was 36.2 and 35.8% for *A. orana* and *B. brassicae* genome, respectively and these values agree with those found for many other insects: for example with 35.9% for *Antheraea pernyi*, 35.1% for *Galleria melonella* (Ooka and Neulat-Portier, 1969), 35.2% for *Hyalophora cecropia* (Wyatt and Linzen, 1965), 37.4% for mulberry silk moth pupae (Gumilevskaya and Sisakyan, 1961), 36% for *Drosophila funebris* (Laird and McCarthy, 1969).

The reassociation data based upon hyperchromicity (Figs. 3 and 4) showed that the *A. orana* and the *B. brassicae* genome differ in their kinetic complexity. A haploid *A. orana* cell has a DNA equivalent to 4.2×10^{10} and that of *B. brassicae* was found to be two times larger than those of *A. orana*, viz 8.4×10^{10} Daltons. The genomes differ in size but both are small and show a remarkable similarity in the extent of intragenome homology. Estimations made from the measurements in Figs. 3 and 4 suggest, that perhaps 90% of the *A. orana* and 91% of the *B. brassicae* DNA nucleotide sequences consist of unique sequences. Estimations from extrapolation of the slow fraction showed that a small fraction 10 and 9% for the *A. orana* and the *B. brassicae* genome respectively, reassociated more rapidly than was expected for a second-order reaction, which suggests the presence of repeated sequences in the genome of both insects. The size and the relative simplicity of the *A. orana* and the *B. brassicae* genome are similar to those of genomes of many other invertebrates, for example the genome of the genus *Drosophila* (Laird and McCarthy, 1969) and of three bee species (Jordan and Brosemer, 1974). However, this similarity is not characteristic for all invertebrates, since sea urchin DNA, for example, shows a high amount of intragenome homology (Britten and Kohne, 1968). The limited intragenome homology also is in contrast to the rapid reassociating fraction observed with DNAs of mammals (Britten and Kohne, 1968).

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V ACTIVATION OF LATENT VIRUS INFECTIONS IN LARVAE OF *ADOXOPHYES ORANA* (LEPIDOPTERA: TORTRICIDAE) AND OF *BARATHRA BRASSICAE* (LEPIDOPTERA: NOCTUIDAE) BY FOREIGN POLYHEDRA

SUMMARY

The number of larvae containing polyhedra increased when larvae of *Adoxophyes orana* and of *Barathra brassicae* were fed on polyhedra of nuclear polyhedrosis virus (NPV) of the reciprocal species. Comparison of restriction endonuclease *Eco* RI cleavage patterns of DNA isolated from polyhedra used as inocula and from polyhedra obtained after cross-inoculation showed that cross--infection did not occur. The observations indicate that latent viruses were activated in both insects. Activation of the *A. orana* latent NPV with polyhedra of a cytoplasmic polyhedrosis virus (CPV) of *B. brassicae*, and cross-inoculation with an extract prepared from healthy larvae indicated that an activating agent does not have to be a component of a nuclear polyhedra.

INTRODUCTION

In a cross-inoculation experiment in the field, Ponsen and de Jong (1964) and Ponsen (1966) used a singly embedded nuclear polyhedrosis virus (SE-NPV) of *Adoxophyes orana* and a multiply embedded nuclear polyhedrosis virus (ME-NPV) of *Barathra brassicae*. They observed that after inoculation of *A. orana* with ME-NPV of *B. brassicae*, the replicated virus was singly embedded in the polyhedral matrix and that after inoculation of *B. brassicae* with SE-NPV of *A. orana* the replicated virus multiply was embedded in the polyhedral matrix. In another cross-inoculation experiment, they also used a cytoplasmic polyhedrosis virus (CPV) of *B. brassicae* for cross-inoculation. Because 90% of the population died after cross-inoculation, they concluded that the SE-NPV of *A. orana* is pathogenic for *B. brassicae* and that the ME-NPV and the CPV of *B. brassicae* are pathogenic for *A. orana*. But they did not establish whether these viruses are cross-infective or whether they activate latent virus infections. However, their observations were of interest from a practical as well as from a theoretical point of view. If these viruses are cross-infective, both insects might be controlled in practice by nuclear or cytoplasmic polyhedra of *B. brassicae*. As *B. brassicae* is large and can be easily reared on an artificial diet, large quantities of *B. brassicae* polyhedra can be produced for application in the field. The insects in the field can be easily contaminated by their own virus (Elmore and Howland, 1964) and they can also be carriers of a latent virus infection (Tanada et al., 1964). Therefore, cross-inoculation experiments in our study were carried out in the laboratory under aseptic rearing conditions with insects reared from surface sterilized eggs.

In the present study, DNA isolated from polyhedra used as inocula and that from polyhedra obtained after cross-inoculation were analysed by cleavage with restriction endonuclease *Eco* RI and then compared to determine whether the nuclear polyhedra of *A. orana* and of *B. brassicae* were cross-infective or activated latent virus infections. After activation of latent virus infections was established, the separate polyhedral components as well as an extract prepared from healthy larvae were used for cross-inoculation experiments, to determine the agent responsible for activation.

MATERIAL AND METHODS

Insects. *A. orana* eggs were supplied by the Laboratory of Entomology, Agricultural University, Wageningen. *B. brassicae* supposedly having a latent

infection of nuclear polyhedrosis virus (NPV) were obtained from Dr. L. Varjas, Research Institute for Plant Protection, Budapest and *B. brassicae* supposedly having a latent infection of cytoplasmic polyhedrosis virus (CPV) were obtained from Dr. Bathon, Darmstadt, Germany.

Polyhedra. The nuclear polyhedra of *A. orana* were obtained from Dr. M.B. Ponsen of our laboratory. The nuclear polyhedra of *B. brassicae* were obtained from Dr. L.P.S. van der Geest, Laboratory of Entomology, University of Amsterdam. The cytoplasmic polyhedra of *B. brassicae* were isolated in our laboratory, from spontaneously diseased larvae obtained from Mr. Wijtebeek, Philips Duphar Laboratory, Weesp.

Rearing of insects. *A. orana* and *B. brassicae* oviposited in cylindrical cages of paper (volume about 3 l). Eggs were laid on the sides of these cages. Every two or three days the cages were cut in strips to collect the eggs and the moths were placed in a new cage. The eggs were surface sterilized with 10% formaldehyde for 40 min (Paschke, 1964). After being washed for 1 hr under running water, the eggs were dried at room temperature. The embryonic development of the eggs took place in Petri dishes near a moistened piece of cotton-wool. The *A. orana* larvae were reared in tubes (high 5 cm, diameter 1.5 cm) on an artificial diet developed by Adkisson et al. (1960). The *B. brassicae* larvae were mass-reared in 2-litre preserving jars (20 larvae per jar) on an artificial diet developed by Nagy (1970). Both insects were reared at 20°C, a relative humidity of 70% and 12 hr of light per day.

Polyhedral inocula. Polyhedra were purified by differential centrifugation according to the procedure of Van der Geest (1968). Filtered homogenates prepared from frozen or freshly collected diseased larvae were centrifuged at 3,000*g* for 30 min at 4°C. The sedimented polyhedra were suspended in distilled water and 10 ml suspension (about 10 mg polyhedra/ml) was layered on 20 ml sucrose solution (61.7% w/w) in SW-25 rotor tubes. After centrifugation at 8,000*g* for 30 min at 4°C, the polyhedra accumulated at the water-sugar interface. The polyhedra were collected and resuspended in distilled water; and the suspension was placed on top of a sucrose solution (43% w/w). Polyhedra were sedimented by centrifuging at 8,000*g* for 30 min at 4°C. The polyhedra were washed free of sucrose by two cycles of centrifugation at 5,000*g* for 30 min at 4°C and then stored in distilled water at 4°C until they were used for preparation of inocula or for purification of virus or DNA. Polyhedral inocula were prepared by dilution of purified polyhedra in 0.05 M Tris-HCl, *p*H 7.5 to a concentration of optical density 2 at 260 nm (about 1 x 10^{8} of polyhedra/ml).

Virus inocula. To purify the virus, the method of Summers and Paschke (1970) was modified. The virus particles were extracted from polyhedra of *A. orana* by exposing them to 0.03 M sodium carbonate plus 0.05 M NaCl (*p*H 10.5) for 30 min at 18°C and those of *B. brassicae* in 0.015 M sodium carbonate (*p*H 10.3) for 30 min at 18°C. The virus particles were separated from the polyhedral proteins by subsequent centrifugation in a 10-40% sucrose gradient. Centrifuging at 23,000 rpm in a SW-25 rotor for 30 min at 4°C sedimented the virus particles in a band and the polyhedral proteins stayed in the fraction on the top of the gradient. The virus particles were collected with a drop collection unit, Model 195 obtained from ISCO. For use as inocula the viruses were diluted in 0.05 M Tris-HCl, *p*H 7.5 to a concentration of optical density 2 at 260 nm.

Polyhedral proteins inocula. The top fraction of the sucrose gradient after virus purification was used for preparation of polyhedral proteins inocula. The solution was dialysed against 0.05 M Tris-HCl, *p*H 7.5 and diluted to a concentration of optical density 2 at 280 nm.

Extracts from healthy larvae. Filtrates from homogenated healthy 4th instar larvae were centrifuged 3 times in a SW-50 rotor at 40,000 rpm for 2 hr at 4°C. Pellets were removed and the freshly prepared supernatants were used as inoculum.

Inoculation of larvae. Inocula were administered to late 3rd and early 4th instar larvae on leaves of *Vicia faba* var. "3 x wit". The seeds were surface sterilized with 3% hydrogen peroxide for 10 min. The plants were reared in pots filled with soil sterilized by steam. The pots were placed in a rearing chamber (20-22°C) where any contamination of plants was prevented. The leaves used had a surface area of about 12 cm^2 and the plants were about 14 days old. Before use the freshly picked leaves were washed with sterilized demineralized water. After washing the slightly damp leaves were placed in Petri dishes on ice. Here they were smeared with inocula, when necessary cut in small disks and kept until they were put into preserving jars or into rearing tubes. About 100 µl of inocula (polyhedra, virus or larvae extract) was smeared on each leaf. The leaves used for inoculation of control larvae were smeared with about 100 µl of 0.05 M Tris-HCl buffer. Larvae of *B. brassicae* were inoculated by giving them leaves smeared with inocula. Larvae of *A. orana* were inoculated by giving them smeared leaf disks (about 3 x 3 mm).

Purification of DNA. A modification of the methods of Gafford and Randal (1967) and Marmur (1961) was used. The virus particles were resuspended in 8 volumes of 1 x SSC (0.15 M NaCl plus 0.015 M sodium citrate, *p*H 7.3) plus 10 mM EDTA and two volumes of 10% sodium dodecylsulphate. Then they were incubated at 60°C for 30 min.

Sufficient 5 M NaCl was added to give a final concentration of 1 M. The suspension was stored overnight at 4°C. After approximately 16 hr at 4°C, the suspension was centrifuged at 15,000*g* to remove the precipitate. The solution was layered onto 2.3 M CsCl and centrifuged in a SW-50 rotor at 30,000 rpm at 25°C. After 24-36 hr centrifugation, the tubes were fractionated with a drop collection unit, Model 195 obtained from ISCO. The DNA fraction was dialysed against 0.02 M Tris-HCl, 3 mM EDTA, *p*H 8.5.

Analysis of DNA by cleavage with restriction endonuclease Eco RI. DNA in 20 mM Tris-HCl plus 1 mM EDTA (*p*H 7.5) was cleaved by restriction endonuclease *Eco* RI and afterwards analysed by electrophoresis on agarose gels as described by Sussenbach and Kuijk (1977).

Light microscopy. Fat body and the gut of *A. orana* and *B. brassicae* larvae smeared on an object slide were dried at room temperature and examined for presence of polyhedra at x 1,000 magnification.

Electron microscopy. Purified polyhedra were prepared for electron microscopy by exposing them to 0.03 M Na_2CO_3 for 15 min, followed by 10-fold dilution with water in order to achieve partial degradation of the polyhedral matrix. The polyhedra were negatively stained with 2% potassium phosphotungstic acid (*p*H 6.8). The electron micrographs were taken with an Elmiscop 101 electron microscope at 80 kV.

Scanning electron microscopy. The wet polyhedra or squashed fat cells and the gut, spread uniformally on specimen mounts were left to dry. The dried polyhedra (or squashed fat-body) were coated with evaporated carbon and gold before being observed in the scanning electron microscope at x 10,000 magnification.

RESULTS

Cross-inoculation with nuclear polyhedra of A. orana and of B. brassicae

The nuclear polyhedra of *A. orana* (SE-NPV) and of *B. brassicae* (ME-NPV) were used for cross-inoculation of 500 larvae of *A. orana* and 500 larvae of *B. brassicae* (suspected of latent infection of NPV). Fifteen days after inoculation, squashed fat-body and the gut of 50 larvae of each species were examined under the light microscope for presence of polyhedra. From each larva one squash preparation was made. The results are shown in Table 1. After inoculation with foreign polyhedra, the number of larvae containing polyhedra increased in both insects when compared with the control. The remaining 450 larvae were reared

Table 1. Number of *A. orana* and *B. brassicae* larvae (from Hungary) containing polyhedra, 15 days after inoculation with foreign nuclear polyhedra.

Larvae of	Inoculated with	Number of larvae			Significance level of the effect of inoculation
		Examined	Containing polyhedra	Without polyhedra	
A. orana	Nuclear polyhedra of *B. brassicae*	150	12	138	0.0359
	Tris-HCl, 0.05 M	150	4	146	
B. brassicae	Nuclear polyhedra of *A. orana*	150	24	126	0.0002
	Tris-HCl, 0.05 M	150	5	145	

Each treatment group (of each experiment) consisted of 500 larvae. Larvae of *B. brassicae* used in this study, were suspected of latent virus infection of NPV. Fifteen days after inoculation, the squashed fat-body and the gut of 50 larvae of each treatment group were examined for presence of polyhedra under the light microscope. The number of control larvae containing polyhedra represent the number of larvae in the "healthy" population carrying homologous latent virus in the form of polyhedra. The actual number of carriers is higher but the other forms of latent virus infection cannot be observed with light microscope. The number of larvae examined (150) is the sum of three separate experiments (3 x 50). Because the number of larvae containing polyhedra after cross-inoculation of each experiment was nearly identical, there was no reason to calculate the significance level for each experiment separately. The significance of the effect of treatment was calculated by Fisher's exact probability test with a Normal approximation for the calculation of the critical level.

until they died as result of polyhedrosis, or pupated. The mortality of cross--inoculated larvae as well as control larvae was lower than we expected from the results represented in Table 1. About 6% of *A. orana* larvae and about 10% of *B. brassicae* larvae of the cross-inoculated group and about 1-2% of control larvae of both insects died of polyhedrosis. Those larvae that died from polyhedrosis were used for purification of polyhedra. Virus obtained from these polyhedra was compared by electron microscopy with the virus obtained from polyhedra used as inocula and with that of control larvae. It was observed that after inoculation of *A. orana* with polyhedra of *B. brassicae*, the replicated virus was singly embedded in polyhedral matrix (Fig. 1A,B) and after inoculation of *B. brassicae* with polyhedra of *A. orana*, the replicated virus was multiply embedded in polyhedral matrix (Fig. 2A,B). It was also shown that the activated virus was homologous with that obtained from polyhedra of control larvae. These observations were in agreement with those of Ponsen and De Jong (1964). As the cross--inoculation was successful, DNA isolated from polyhedra used as inocula and that from polyhedra obtained after cross-inoculation were compared to determine whether this cross-inoculation resulted in an activation of latent virus infections or in cross-inoculation.

Comparison of DNA isolated from polyhedra used as inocula with that from polyhedra obtained after cross-inoculation, after cleavage by restriction endonuclease Eco RI

Because DNA of the *A. orana* polyhedral inoculum and DNA of the *B. brassicae* polyhedral inoculum cleaved by *Eco* RI give quite different band patterns after electrophoresis in agarose gels, it was possible to distinguish them from each other as well as compare them with DNA isolated from polyhedra obtained after cross-inoculation. As seen from Fig. 3 (A,C and B,D), the cleavage patterns of DNA of the polyhedral inocula and DNA of the polyhedra obtained after cross--inoculation are not identical. However the cleavage pattern of DNA of the polyhedral inoculum and DNA of the activated virus of the homologous insect are identical (Fig. 3A,D and B,C). The DNA of activated virus was also homologous with that purified from virus of the control larvae.

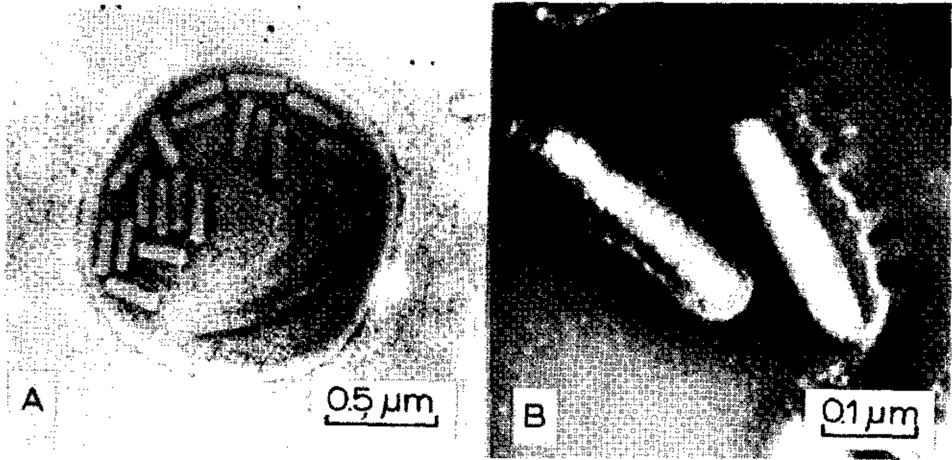

Fig. 1. Polyhedra with singly embedded virus particles in polyhedral matrix, found in *A. orana* larvae after inoculation with nuclear polyhedra of *B. brassicae*. Partially disrupted polyheder (A), detail of disintegrated virus particles (B).

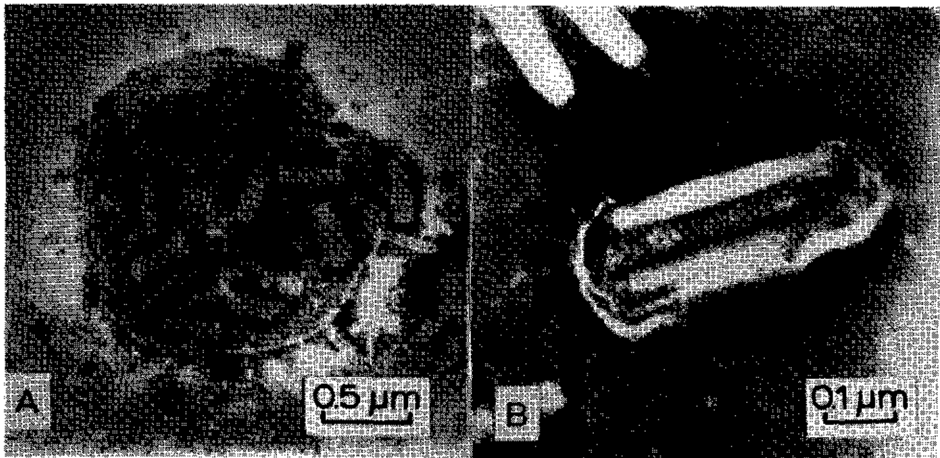

Fig. 2. Polyhedra with multiply embedded virus particles in polyhedral matrix found in *B. brassicae* larvae (from Hungary) after inoculation with nuclear polyhedra of *A. orana*. Partially disrupted polyheder by alkali treatment (A), detail of a disintegrated virus particle (B).

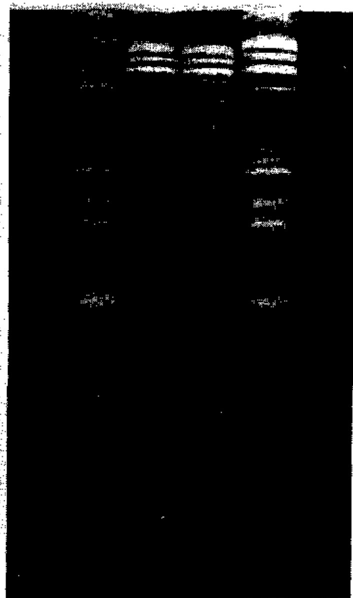

Fig. 3. Patterns of NPV-DNAs cleaved by restriction endonuclease *Eco* RI in 1.4% agarose gels. DNA of the *A. orana* polyhedral inoculum (A), DNA of the *B. brassicae* polyhedral inoculum (B), DNA of polyhedra obtained from *B. brassicae* (from Hungary) after inoculation with nuclear polyhedra of *A. orana* (C), DNA of polyhedra obtained from *A. orana* after inoculation with nuclear polyhedra of *B. brassicae* (D).

Cross-inoculation with nuclear polyhedra of A. orana and of cytoplasmic polyhedra of B. brassicae

After activation of latent NPV with foreign nuclear polyhedra was established, another cross-inoculation was made to demonstrate whether the cytoplasmic polyhedra of *B. brassicae* are cross-infective or act as an activating agent of a latent virus infection. In this experiment 500 larvae of *A. orana* were inoculated with cytoplasmic polyhedra of *B. brassicae* and 500 larvae of *B. brassicae* (suspected of a latent infection of CPV) with nuclear polyhedra of *A. orana*. Fifteen days after inoculation, squashed fat-body and the gut of 50 larvae of each species were examined for the presence of nuclear or cytoplasmic polyhedra. Because nuclear polyhedra of *A. orana* differ in form from the cytoplasmic polyhedra of *B. brassicae* (Ponsen et al., 1965), scanning microscopy was used to determine the shape of the polyhedra formed after cross-inoculation. As shown in Table 2, the number of *A. orana* larvae containing nuclear polyhedra increased slightly, after inoculating them with cytoplasmic polyhedra of *B. brassicae* and the number of *B. brassicae* larvae containing cytoplasmic polyhedra was high,

Table 2. Number of *A. orana* larvae containing nuclear polyhedra and *B. brassicae* larvae (from Germany) containing cytoplasmic polyhedra, 15 days after cross-inoculation.

Larvae of	Inoculated with	Number of larvae			Significance level of the effect of treatment
		Examined	Containing polyhedra	Without polyhedra	
A. orana	Cytoplasmic polyhedra of *B. brassicae*	150	10	140	0.1446
	Tris-HCl, 0.05 M	150	5	145	
B. brassicae	Nuclear polyhedra of *A. orana*	150	42	108	0.0001
	Tris-HCl, 0.05 M	150	9	141	

Each treatment group (of each experiment) consisted of 500 larvae. Fifteen days after cross-inoculation, fat-body and the gut of 50 larvae of each group were examined under the light microscope. The number of control larvae containing polyhedra represent the number of larvae in the "healthy" population carrying homologous latent virus in the form of polyhedra. The actual number of carriers is higher but the other forms of latent virus infection cannot be observed with light microscope. The number of larvae examined (150) is the sum of three separate experiments (3 x 50). Because the number of larvae containing polyhedra after cross-inoculation of each experiment was nearly identical, there was no reason to calculate the significance level for each experiment separately. The significance of the effect of treatment was calculated by Fisher's exact probability test with a Normal approximation for the calculation of the critical level.

after inoculating them with nuclear polyhedra of *A. orana*. The remaining 450 larvae were reared until they died from polyhedrosis or pupated. About 6% of *A. orana* and about 15% of *B. brassicae* larvae of the cross-inoculated group and about 1-2% of control larvae of both insects died of polyhedrosis. Polyhedra isolated from larvae that died after cross-inoculation were examined for the presence of polyhedra with the same shape as those used as inocula. In the preparations of the polyhedra purified from *A. orana* larvae, no cytoplasmic polyhedra were seen, instead nuclear polyhedra were observed, Fig. 4. In the preparations of the polyhedra purified from *B. brassicae*, no nuclear polyhedra were seen, instead cytoplasmic polyhedra were observed (Fig. 5). Scanning microscopy also showed that the activated polyhedra are homologous with those isolated from the control group.

Agent responsible for activation of latent virus infections

The agent responsible for the activation of latent virus infection observed in *A. orana* and *B. brassicae* larvae could be the virus, polyhedral protein or an unknown component occurring in larvae that can be associated with polyhedra. Therefore, viruses, polyhedral protein and an extract from healthy larvae of *A. orana* and of *B. brassicae* were used to cross-inoculate 500 3rd and early 4th instar larvae of *A. orana* and 500 larvae of *B. brassicae* (suspected of a latent infection of NPV). Fifteen days after inoculation, squashed fat-body and the gut of 50 larvae of each species were examined under the light microscope for presence of polyhedra. From each larva one squash preparation was made. No significant increase of larvae containing polyhedra was observed when viruses or polyhedral protein (prepared by alkali treatment) were used as inoculum. However the number of *B. brassicae* larvae containing polyhedra was larger when an extract prepared from healthy *A. orana* larvae was used for cross-inoculation. The number of *A. orana* larvae containing polyhedra after inoculation with an extract prepared from healthy *B. brassicae* larvae seemed to increase slightly, but not significantly. The results of these experiments are given in Table 3. They indicate that a component occurring in healthy larvae can activate the latent virus infections rather than the constituents of the polyhedra.

DISCUSSION

For a number of years some insect virologists have thought that insect viruses are species-specific and that cross-infection does not occur (Smith, 1967). This

Table 3. Number of *A. orana* and *B. brassicae* (from Hungary) larvae containing polyhedra, after cross-inoculation with NPV, inclusion body proteins and extract of healthy larvae.

Larvae of	Inoculated with	Number of larvae			Significance level of the effect of treatment
		Examined	Containing polyhedra	Without polyhedra	
A. orana	Virus	150	5	145	NS[1]
	Polyhedral protein	150	4	146	NS
	Extract of *B. brassicae* larvae	150	8	142	NS
	Tris-HCl, 0.05 M	150	5	145	
B. brassicae	Virus	150	6	144	NS
	Polyhedral protein	150	9	141	NS
	Extract of *A. orana* larvae	150	18	132	0.0183
	Tris-HCl, 0.05 M	150	7	143	

Third and early 4th instar larvae (reared from surface sterilized eggs) were given leaves smeared with the following inocula: virus, optical density of 2.0 at 260 nm, polyhedral proteins, optical density of 2.0 at 280 nm and undiluted extract prepared from healthy larvae. Each treatment group (of each experiment) consisted of 500 larvae. Fifteen days after inoculation, fat body and the gut of 50 larvae of each group were examined under the light microscope. The number of larvae examined (150) is the sum of three separate experiments (3 x 50). Because the number of larvae containing polyhedra after cross-inoculation of each experiment was nearly identical, there was no reason to calculate the significance level for each experiment separately. The significance of the effect of treatments was calculated by Fisher's exact probability test with a Normal approximation for the critical level.

[1] NS; not significant.

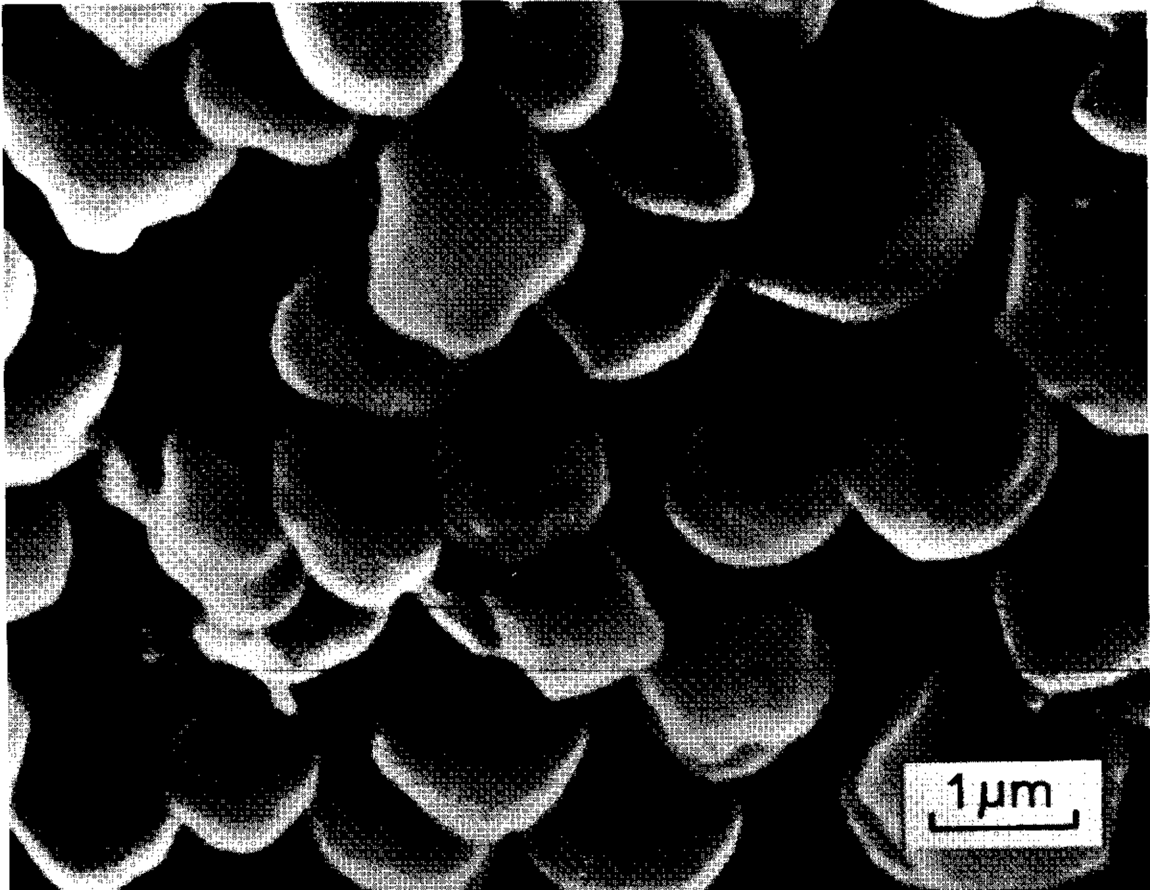

Fig. 4. Polyhedra found in *A. orana* after inoculation with cytoplasmic polyhedra of *B. brassicae*.

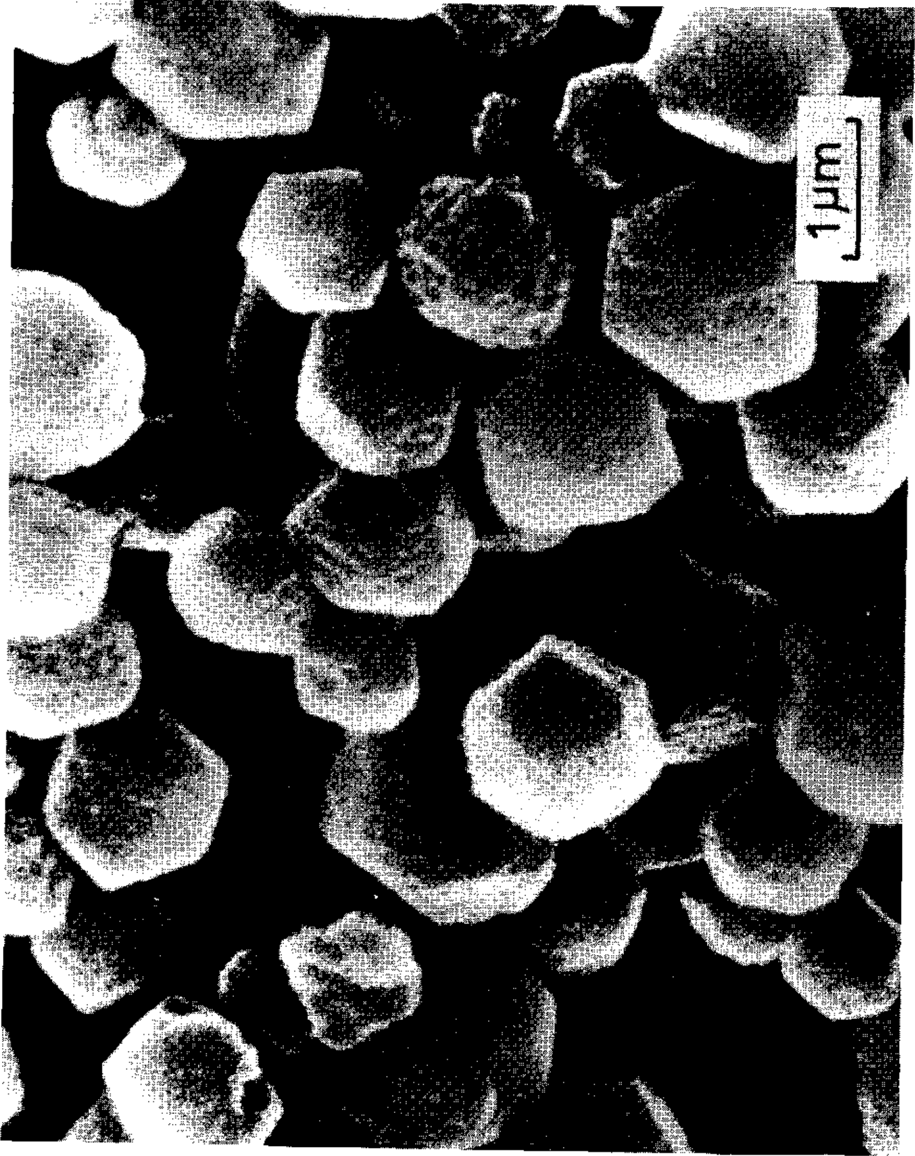

Fig. 5. Polyhedra found in *B. brassicae* larvae (from Germany) after inoculation with nuclear polyhedra of *A. orana*.

idea was indirectly supported by Smith and Rivers (1956), Krieg (1957), Smith (1963) and Karpov (1963), who discovered that foreign polyhedra can activate latent virus infections in insect larvae. However, they did not study which factor caused the activation. Longworth and Cunningham (1968), who discovered that NPV of *Aglais urticae* and of *Portheria dispar* are activated by cross--inoculation and Grace (1962), who showed that CPV of *Antheraea eucalipti* can be activated by nuclear polyhedra of *Bombyx mori*, suggested that the polyhedral protein of the activating virus was responsible for activation of the latent viruses. On the other hand, evidence provided by Steinhaus (1953), Smith et al. (1961), Sidor (1960), Smirnoff (1963), Stairs (1964), Vail and Jay (1973) and Stairs and Lynn (1974) makes it clear that insect viruses do not have to be species-specific and that cross-infections by foreign nuclear polyhedra may occur as well.

In the past when singly and multiply embedded NPVs were used for cross--inoculation, light (Gershenson, 1960) or electron microscopy (Smith et al., 1959; Sidor, 1960; Ponsen and De Jong, 1964) was applied to determine whether these viruses were cross-infective or inducing agents of latent virus infections. However, light microscopy can give erratic results as one has to rely on the shape of the polyhedra. It remains uncertain whether the shape of the polyhedra is virus specific (Stairs et al., 1966; Shigematsu and Suzuki, 1971). By electron microscopy, it is possible to distinguish the SE-NPV from ME-NPV (Figs. 1 and 2) but it is not possible to establish whether cross-infection or activation of latent virus infections occurs because an insect can be infected with two morphologically different NPVs (Heimpel and Adams, 1966) and because it is not yet known why some NPVs are SE and some ME. Therefore, in the present study genetic material was analysed to prove unequivocally that NPVs of *A. orana* and of *B. brassicae* are not cross-infective but activating agents of latent virus infections (Fig. 3). The comparison of polyhedral DNA by cleavage with restriction enzyme *Eco* RI was chosen because the restriction enzymes are highly specific endonucleases that cleave native DNA at a limited number of sites, breaking both chains of the duplex (Meselson and Yuan, 1968; Takano et al., 1968).

In our experiments the number of larvae containing polyhedra after cross--inoculation (Table 1) was much lower than the mortality rate found by Ponsen and De Jong (1964), who reported that 90% of the population died after cross--inoculation. Moreover, not all larvae containing polyhedra die of polyhedrosis. The percentage of dead larvae after cross-inoculation with foreign polyhedra was about 6% for *A. orana* and about 10 or 15% for *B. brassicae* larvae carrying a

latent NPV or CPV, respectively. This difference in results is most probably caused by rearing conditions. The experiments of Ponsen and De Jong were done under field conditions where the insects can very easily be contaminated by their own polyhedra while our experiments were started with 10th generation reared from repeatedly surface-sterilized eggs under strong aseptic conditions.

To imitate the method of inoculation used by Ponsen and De Jong (1964) as closely as possible, the inocula for the late 3rd and early 4th instar larvae were administered on leaves of *Vicia faba* var. "3 x wit". Larvae of both insects preferred the leaves of *Vicia faba* to an artificial diet. Therefore these leaves were suitable for giving the inocula to the larvae at an appropriate time. Comparison of the number of control larvae containing polyhedra with the number of uninoculated larvae containing polyhedra (unpublished results) showed that the leaves smeared with 0.05 M Tris-HCl buffer had no effect on the activation of latent virus infection.

The second problem was to study whether the cytoplasmic polyhedra of *B. brassicae* are cross-infective or activate latent virus infection. Here scanning electron microscopy can readily distinguish the cubical cytoplasmic polyhedra of *B. brassicae* from spherical nuclear polyhedra of *A. orana* (Ponsen et al., 1965). As seen from results obtained in Table 2 and Figs. 4 and 5, the cytoplasmic polyhedra of *B. brassicae* are not cross-infective and also they, as the nuclear polyhedra of *B. brassicae*, cannot be used for biological control of *A. orana* in the field.

The third point was to study what actually activated latent virus infections in insects. Longworth and Cunningham (1968) and Grace (1962) suggested that the agent responsible for activation is the polyhedral protein. However they did not consider other agents, such as a protein which rather than the virus particles, can be occluded in the polyhedra. This agent can originate from the larvae (probably coded by host DNA) and can be associated with virus particles rather than with polyhedral protein. Thus, it is complicated to determine whether one of the constituents of the nuclear polyhedra acts as an activating agent of a latent virus infection, especially as this agent can be destroyed during purification of virus particles and polyhedral protein by alkali treatment as indicated in Table 3. Moreover, these components can be contaminated by agents originated from the larvae and by each other's protein.

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VI TRANSMISSION OF NUCLEAR POLYHEDROSIS VIRUS TO THE PROGENY OF *ADOXOPHYES ORANA* AND OF *BARATHRA BRASSICAE*

SUMMARY

Experiments showed that nuclear polyhedrosis virus (NPV) of *Adoxophyes orana* and of *Barathra brassicae* can be transmitted to the progeny of these insects inside the eggs (transovarial) and by surface contamination of the eggs (transovum). The transmission of NPV in both insects was examined by light microscopy of the polyhedra present in fat cells of larvae. The way in which NPV was transmitted was determined by comparing the number of polyhedra carriers descended from surface sterilized eggs with that from untreated eggs. There were more polyhedra carriers in insects reared from untreated eggs than in insects reared from surface sterilized eggs. Thus transovum as well as transovarial transmission was indicated. The presence of virus in both groups of insects was also confirmed by infectivity experiments. Transovarial transmission was indicated by hybridization experiments of viral DNA with DNA isolated from fourth instar larvae (reared from surface sterilized eggs) in which viral sequences were revealed.

INTRODUCTION

The generation-to-generation spread of nuclear polyhedrosis viruses (NPVs) has been investigated extensively. Transovum transmission, which is due to surface contamination of eggs has been studied by Tanada (1964), Martignoni and Milstead (1962), Rivers (1967), Knipling (1960), Elmore and Howland (1964), Vail and Hall (1969) and Hamm and Young (1974). They found that migratory movement of infected larvae is probably instrumental in the spread of virus by transovum transmission in the field. Many other workers have reported that transovarial (within the egg) transmission may occur as well: Hukuhara (1962), Aruga and Nagashima (1962), Tanada et al. (1964), Smith and Wyckoff (1951), Kislev et al. (1971), David and Taylor (1976). However, conclusive experiments supporting this idea are lacking. For instance, Yamafuji et al. (1966) suggested that the polyhedral previral genome may be located in the DNA of the host cell but they gave no evidence that the viral genome was indeed transmitted to the progeny by association with the host genome.

This paper describes experiments that were designed to study generation-to--generation transmission of nuclear polyhedrosis virus of *Adoxophyes orana* and of *Barathra brassicae*. Transovum transmission was examined by comparing the number of polyhedra carriers descended from surface sterilized eggs with that from untreated eggs. Transovarial transmission was investigated by reassociation experiments of ^{32}P-labelled virus DNA with host DNA prepared from "healthy" larvae reared from surface sterilized eggs.

MATERIALS AND METHODS

Insects. *Barathra brassicae* cultures supposedly having a latent nuclear polyhedrosis virus were obtained from Dr. L. Varjas, Research Institute for Plant Protection, Budapest. Larvae without latent virus infection were collected in 1975 in an allotment in Wageningen. *Adoxophyes orana* eggs were obtained from Laboratory of Entomology, Agricultural University, Wageningen.

Viruses. The NPV of *B. brassicae* was obtained from Dr. L.P.S. van der Geest, Laboratory of Entomology, University of Amsterdam. The NPV of *A. orana* was obtained from Dr. M.B. Ponsen, Laboratory of Virology, Agricultural University, Wageningen.

Diets. The composition and the preparation of *B. brassicae* diet was described by Nagy (1970): 20 g agar, 40 g saccharose, 150 g wheat germ, 100 g lucerne and

40 g yeast were added to 1,000 ml of water. After boiling for 20 min and cooling to 50°C, 5 ml of glacial acetic acid and 4 g methyl-p-hydroxybenzoate were added. The *A. orana* diet is a modification of a diet described by Adkisson et al. (1960): 35 g casein, 30 g wheat germ, 35 g saccharose, 5 g Wesson's salt, 20 g agar and 20 ml of choline chloride were added to 850 ml water. After this mixture was boiled for 20 min, 8 g ascorbic acid, 8 g Nipagine and 5 ml corn oil were added.

Rearing of insects. The method used to rear *B. brassicae* was a modification of that of Nagy (1970). Oviposition took place in standing cylindrical cages of paper (volume about 3 litres) that were covered at both ends with filter paper and a Petri dish. Moistened cotton-wool provided water for the moths. Not more than 20 moths (10 ♂ and 10 ♀) were placed in a cage. Every two or three days the paper cylinders were cut in strips to collect the eggs laid and the moths were placed in a new cylinder. During embryonic development, eggs were kept in Petri dishes near a moistened piece of cotton-wool. Eggs were surface sterilized by processing with 10% formaldehyde solution for 1 hr (Paschke, 1964). After washing for 1 hr under running water, the eggs were dried at room temperature and checked for the presence of polyhedra by scanning electron microscope. The strips of paper with the adhering egg clusters were placed in a preserving jar with diet smeared on its sides. The jars were closed with cheesecloth. During growth larvae needed no special attention. Fully developed larvae were dark brown. At this stage, larvae were put in boxes provided with a mixture of peat and moist sand and a little of the diet on a plastic sheet. Dark brown pupae were transferred to glass jars with crumpled sheets of filter paper on the bottom. The emerged adults were collected and put in vials. After selection and sexing, the moths were placed in the oviposition cylinders.

For oviposition of *A. orana* two cylinders were used: one cylinder with moths from surface sterilized eggs and one with moths from untreated eggs. The eggs were collected in the same way as for *B. brassicae*. Eggs laid by moths from the sterilized eggs were surface sterilized and those laid by moths from untreated eggs were not surface sterilized. During embryonic development, eggs were kept in glass tubes (high 12 cm, diameter 3 cm). Hatching larvae were transferred by brush into rearing tubes (high 5 cm, diameter 1.5 cm), filled with the diet to one third of their height. A tube contained only one larva and was closed with a plug of cotton-wool. The fully developed pupae were collected from these tubes and put in vials. After selecting and sexing, moths were placed in the oviposition cylinders.

Both insect species in all development stages were reared in Sherer chambers at 23°C, 70% relative humidity and 12 hr light per day.

Preparation of polyhedra. The polyhedra were purified as described by Van der Geest (1968). Filtered homogenates from larvae infected by their own NPV, were centrifuged at 3,000*g* for 30 min. The sedimented polyhedra were suspended in water and 10 ml of suspension (10 mg/ml) was layered on 20 ml of a sucrose solution 61.7% (w/w). After centrifuging at 8,000*g* for 30 min, the polyhedra were collected from the water-sucrose solution interface. These polyhedra were resuspended in water and a suspension of 10 ml was placed on the top of the sucrose solution 43.9% (w/w). The polyhedra were sedimented by centrifuging at 8,000*g* for 30 min. The sedimented polyhedra were washed free of sucrose by two cycles of centrifugation at 5,000*g* for 30 min and stored in distilled water at $4^{o}C$. The polyhedra were used for purification of DNA or for inoculation of larvae. For use as inocula the polyhedra were diluted with distilled water to an optical density of 2.0 at 260 nm.

Extracts of the development stages of insects. Insects of the development stage in which they stopped their development were used for preparation of extracts which were used as inocula. Thus extracts were obtained from: dead larvae, dead prepupae, dead pupae or pupae whose development was of long duration, moths from such pupae and clusters of eggs laid by moths from pupae with a normal duration of development. The inocula were prepared by centrifugation of filtered extracts at 90,000*g* for 2 hr. The pellets were resuspended in 1 ml water and used as inocula.

Inoculation of larvae. Third and early fourth instar larvae of *B. brassicae* reared in preserving jars (20 larvae per jar of 2 l), were inoculated by giving them leaves of *Vicia faba* var. "3 x wit" smeared with extracts prepared from insects. The third and early fourth instar larvae of *A. orana*, reared individually in rearing tubes, were inoculated by giving them smeared leaf disks.

Virus purification. Virus particles from polyhedra were isolated and purified by the procedure of Summers and Paschke (1970). The polyhedra of *A. orana* were exposed to 0.03 M sodium carbonate plus 0.05 M NaCl (*p*H 10.5) for 30 min at $18^{o}C$ and those of *B. brassicae* to 0.015 M sodium carbonate (*p*H 10.3) for 15 min at $18^{o}C$. The mixtures were then layered on 10-40% (w/v) sucrose in 0.05 M sodium phosphate buffer (*p*H 7.8). Centrifugation was conducted at 23,000 rpm and $4^{o}C$ in SW-27 rotor for 30 min. Virus fractions were collected with a drop-collection unit Model 195 obtained from ISCO and sedimented by centrifugation at 90,000*g* for 60 min. Pellets were resuspended in 0.05 M sodium phosphate buffer (*p*H 7.8) or in 0.15 M NaCl, 0.015 M sodium citrate, *p*H 7.4 (1xSSC) plus 10 mM EDTA, when directly used for purification of DNA.

Purification of virus DNA. DNA was extracted by a combination of methods of Gafford and Randal (1967) and Marmur (1961). The virus was suspended in 8 volumes of 1xSSC plus 10 mM EDTA and 2 volumes of 10% sodium lauryl sarcosinat (SLS). The suspension was heated in a water bath at $60^{o}C$ for 30 min. Then 5 M NaCl was added to a final concentration of 1 M. After approximately 14 hr at $4^{o}C$, the solution was centrifuged for 30 min at 15,000*g* to remove the precipitate. The solution was layered on a solution of caesium chloride (CsCl) in SW-50 rotor tubes and centrifuged at 30,000 rpm for 36 hr at $25^{o}C$. Fractions were collected with a drop-collection unit Model 195 obtained from ISCO. The fraction containing DNA was dialysed against 1xSSC plus 3 mM EDTA (*p*H 7.4).

Isolation of host DNA. The procedure used is a combination and modification of the methods of Gall and Atherton (1974) and Gross et al. (1973). Five g of fourth instar larvae, reared from surface sterilized eggs, were ground at $4^{o}C$ and then solution of 0.05 M Tris-HCl, 0.025 M KCl, 0.005 M magnesium acetate and 0.35 M sucrose (*p*H 7.6) was added. The filtered suspension was centrifuged at 5,900*g* for 10 min. The pellet was suspended in 0.05 M Tris-HCl buffer, 0.1 M EDTA, 0.5% β-mercaptoethanol, 0.5% SLS and 300 μg/ml Proteinase K (*p*H 7.7). After incubation for 90 min at $37^{o}C$, SLS was added to a concentration of 2% and the solution was incubated at $60^{o}C$ for 30 min. The viscous solution was stirred with an equal volume of water-saturated phenol for 30 min. The phenol treatment was repeated twice followed by centrifugation at 12,000*g* for 30 min. The aqueous phase containing DNA was dialysed against 1xSSC plus 10 mM EDTA (*p*H 7.4). The crude DNA was treated with pancreatic RNase 150 μg/ml, RNase T_1 495 units/ml and α-Amylase 375 μg/ml for 2 hr at $37^{o}C$. Then Pronase 300 μg/ml was added and the incubation at $37^{o}C$ was continued for 2 hr to reduce the protein content of the solution. The digestion was followed by dialysing the DNA against 0.02 M Tris-HCl buffer plus 10 mM EDTA (*p*H 8.5). Purification was completed by isopycnic centrifugation in CsCl, to separate the host DNA from nonintegrated virus DNA. The (G+C) content of the *A. orana* host DNA and that of *A. orana* NPV-DNA was determined to be 36.2 and 34.5%, respectively. The (G+C) content of the *B. brassicae* genome and of the *B. brassicae* NPV-DNA was determined to be 35.8 and 37%, respectively (Chapter III and IV). Centrifugation was performed in SW-41 rotor tubes at 30,000 rpm for 36 hr at $25^{o}C$. The denser part of the *A. orana* host DNA band and the lighter part of the *B. brassicae* DNA band was repurified by centrifugation in CsCl and afterwards used for reassociation.

Labelling of virus DNA. DNA was labelled to a high specific activity by nick-translation as described by Rigby et al. (1977). The reaction mixture of 50 μl

contained: 200 p mol of α-^{32}P-dATP, 200 p mol α-^{32}P-dCTP (evaporated), 2 µl 0.05 mM dGTP, 2 µl 0.25 mM dTTP, 5 µl 10 x nick-buffer (500 mM Tris-HCl, 50 mM MgCl, 100 mM β-mercaptoethanol, 500 µg/ml Bovine serum albumin), 2 µl NaCl, 25 µl of *A. orana* or *B. brassicae* NPV-DNA concentration 20 µg/ml, 8 µl DNase 10^{-8} g/ml and 1 µl DNA polymerase I. The reactions were carried out at 13.5°C for 90 min. The reaction mixtures were extracted once with phenol and once with a mixture of chloroform-isoamyl alcohol (24 : 1). The mixtures were passed over a G-50 Sephadex column to separate the DNA from the remaining substrates.

Hybridization. Before reannealing, the labelled viral DNA and the unlabelled host DNA were mixed and degraded to nucleotide segments, 200 to 300 bases in length, by boiling in 0.3 N NaOH for 20 min (Sharp et al., 1974). The solutions (500 µl volume) were immediately immersed in ice. After being cooled the mixtures were adjusted to *p*H 6.8 with ice-cold 140 µl sodium phosphate buffer (1 M), 40 µl SLS (10%), 140 µl NaCl (5 M), 158.4 µl water and 21.6 µl HCl (6 N). DNA was renatured by incubation of the fragmented DNA in closed ampules at 68°C. Renaturation was stopped by diluting the samples with 1 ml of ice-cold 0.14 M sodium phosphate buffer (*p*H 6.8). The extent of renaturation was analysed on hydroxylapatite columns at 60°C. Single-stranded DNA was eluted with 0.14 M sodium phosphate buffer and double-stranded DNA with 0.4 M sodium phosphate buffer (Sambrook et al., 1972).

Light microscopy. The fat cells of *A. orana* and *B. brassicae* larvae were smeared on an object slide and dried at room temperature. The preparations were examined for the presence of polyhedra with a light microscope at x 1,000 magnification.

RESULTS

Effect of surface sterilization of Adoxophyes orana eggs on nuclear polyhedrosis virus (NPV) transmission

The effect of surface sterilization of eggs of *A. orana* on transmission of NPV to the progeny was studied by light microscopy of the polyhedra present in fat cells of larvae reared from surface sterilized eggs and from untreated eggs. Larvae of both groups were allowed to grow for 15 days. The larvae tested did not show any symptoms of NPV infection. From each larvae one preparation of fat cells was made. The number of fat-cell preparations containing polyhedra was taken as the number of NPV carriers. The results of light microscopical examination are shown in Table 1. The smaller number of carriers descended from treated eggs

indicates that NPV can be transmitted to the progeny on the surface of eggs as well as inside them.

Table 1. The numbers of the NPV carriers of *A. orana* larvae reared from surface sterilized and untreated eggs

Source of larvae	Number of larvae examined (per experiment)	Average number of larvae containing polyhedra	% of polyhedra carriers
Untreated eggs	200	19	9.5
Surface sterilized eggs	200	6	3.0

The fat cells of 15-day-old *A. orana* larvae were examined microscopically for polyhedra. From each larvae one preparation of fat cells was made. Number of NPV carriers was equal to number of fat-cell preparations containing polyhedra. This experiment was repeated twice.

Besides 400 larvae reared from surface sterilized eggs, 400 larvae from untreated eggs were used to confirm the microscopic observations by infectivity tests. Insects whose development had stopped or was retarded at various stages before maturity were used for preparation of extracts. The preparation of extracts is outlined in Table 2. Polyhedra were seen by electron microscopy in all extracts except those made from eggs. The pellets obtained by centrifuging of these extracts were used to inoculate third and early fourth instar larvae reared from surface sterilized eggs. Mortality was maximal about 14 days after inoculation. Dead larvae and those with symptoms of NPV disease were counted 21 days after inoculation. The results of these experiments are shown in Table 3. They indicate that inocula prepared from dead larvae had the highest infectivity. Inocula prepared from insects originating from surface sterilized eggs contained much less infective material than those from insects originating from untreated eggs. The results of both groups indicated that generation-to-generation transmission as well as transstadial transmission of infective material are possible.

Transmission of NPV in Barathra brassicae reared from surface sterilized eggs

The number of NPV carriers was determined by light microscopy of the polyhedra present in fat cells of 15-day old larvae reared from surface sterilized eggs. Because the *B. brassicae* larvae are large, they were mass-reared in preserving jars. For this reason it was not possible to determine the number of NPV carriers in larvae originated from untreated eggs. The number of fat-cell

Table 2. Scheme of extracts prepared from *A. orana* in various stages of development

Group I Insects reared from untreated eggs		Group II Insects reared from surface sterilized eggs	
Number and development stage of insects	Extract prepared from	Number and development stage of insects	Extract prepared from
400 larvae	13 larvae, dead	400 larvae	3 larvae, dead
387 prepupae	11 prepupae, dead	397 prepupae	5 prepupae, dead
377 pupae	39 pupae, dead or development retarded	392 pupae	18 pupae, dead or development retarded
338 moths	22 moths from pupae of retarded development	374 moths	7 moths from pupae of retarded development
eggs of healthy moths	200 egg clusters	eggs of healthy moths	200 egg clusters

For the experiments we used healthy *A. orana* larvae reared from egg masses laid by females of the same origin. The eggs were divided into two groups. One half was surface sterilized with formaldehyde and the other half was untreated. Both were reared on an aseptic diet under the same light-temperature regime. The adults reared from eggs of both groups were used for production of eggs. The experiments were started after rearing 10 generations under described conditions. At the start of this experiment, there were 400 *A. orana* larvae reared from surface sterilized eggs and 400 larvae from untreated eggs. The preparation of extracts is described in Materials and Methods.

Table 3. Infectivity of extracts prepared from various developmental stages of *A. orana* reared from untreated eggs and from surface sterilized eggs.

Extracts prepared from:	Dead larvae	Dead prepupae	Dead or slowly developing pupae	Moths from slowly developing pupae	Eggs laid by healthy moth	Control
Number of larvae inoculated with extracts of Group I	50	50	50	50	50	50
Number of larvae with symptoms of NPV disease	45	17.5	15	6.5	3	1.5
Number of larvae inoculated with extracts of Group II	50	50	50	50	50	50
Number of larvae with symptoms of NPV disease	27.5	7	2.5	2.5	1	1

Larvae used for inoculation were reared from surface sterilized eggs. Extracts of Group I were prepared from insects reared from untreated eggs. Extracts of Group II were prepared from insects reared from repeatedly surface sterilized eggs. Preparation of extracts is outlined in Table 2 and described in Materials and Methods. As control were taken leaves smeared with water. Number of *A. orana* larvae with symptoms of NPV disease was counted 21 days after inoculation. The results are the average of 2 experiments.

preparations containing polyhedra was taken as the number of nuclear polyhedrosis virus carriers. The results of these studies showed that about 4% of *B. brassicae* larvae reared from surface sterilized eggs contained polyhedra in their fat cells.

In another experiment 400 larvae were used to confirm the occurrence of NPV in *B. brassicae* by infectivity tests. The number of insects used for preparation of extracts is outlined in Table 4. Polyhedra were seen by electron microscopy in all extracts except those prepared from eggs. The pellets obtained by centrifuging of these extracts were used to inoculate third and fourth instar larvae reared from surface sterilized eggs. Mortality was maximal about 12 days after inoculation. Dead or diseased larvae with symptoms of NPV disease were counted 21 days after inoculation. The results of inoculation of third and fourth instar larvae with inocula prepared from various developmental stages of *B. brassicae*

Table 4. Scheme of extracts prepared from various developmental stages of *B. brassicae*

Number and development stage of insects	Extract prepared from
400 larvae	4 larvae, dead
393 prepupae	4 prepupae, dead
392 pupae	14 pupae, dead or development retarded
378 moths	11 moths from pupae of retarded development
eggs of healthy moths	200 clusters

At the start of experiment there were 400 *B. brassicae* larvae (from Hungary), mass reared from surface sterilized eggs. Preparation of extracts is described in Material and Methods.

are given in Table 5. The results indicate that virus material must have been transmitted from generation-to-generation inside the eggs, and that this transmission was followed by transstadial transmission as in *A. orana* insects.

Demonstration of NPV sequences in samples of the A. orana host DNA

The association of *A. orana* NPV-DNA with its host DNA was demonstrated by reassociation of ^{32}P-labelled NPV-DNA with unlabelled host DNA, isolated from 4th instar larvae reared from surface sterilized eggs. The reaction mixture for the study of viral sequences in host DNA contained 1.81×10^{-3} μg of ^{32}P--labelled NPV-DNA and 480 μg of unlabelled *A. orana* host DNA. The negative control contained 1.72×10^{-3} μg of ^{32}P-labelled NPV-DNA with 480 μg of un-

labelled *E. coli* DNA and the positive control 1.62×10^{-3} µg of ^{32}P-labelled NPV-DNA with 0.237 µg of unlabelled NPV-DNA.

In comparison to the reassociation experiment with ^{32}P-labelled NPV-DNA and unlabelled *A. orana* host DNA the positive control experiment contains the equivalent of 0.62 copies of viral DNA per diploid amount of *A. orana* host DNA if the molecular weights of *A. orana* host DNA and NPV-DNA are taken 8.4×10^{10} and 6.7×10^{7}, respectively. The results of the reassociation experiment are shown in Table 6 and Fig. 1. Fig. 1 reveals that with *A. orana* host DNA, the ^{32}P-labelled NPV-DNA reassociates slightly faster than with *E. coli* DNA, indicating that *A. orana* host DNA contains a small amount of NPV-DNA. Comparison of the slopes of the reassociation lines of the *A. orana* host DNA with NPV-DNA and the positive control experiment (Fig. 1) reveals that *A. orana* host DNA contains about 0.03 viral DNA equivalents per diploid cell.

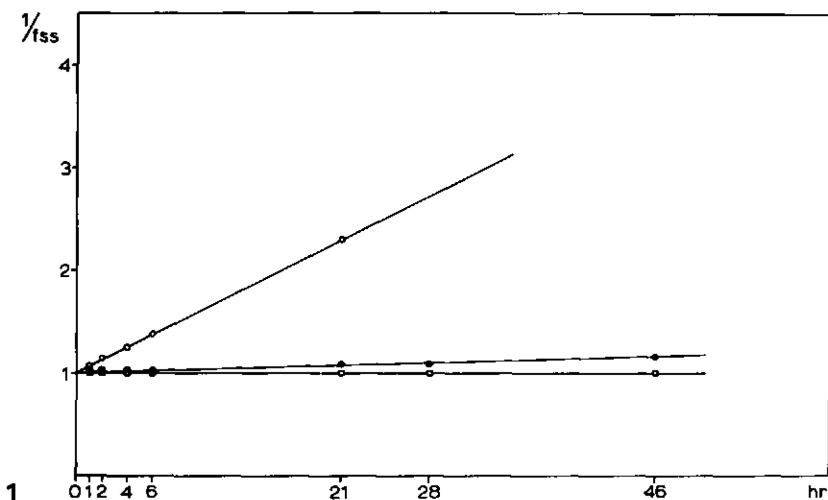

Fig. 1. Reassociation of ^{32}P-labelled *A. orana* NPV-DNA in the presence of *A. orana* host DNA (●—●), *E. coli* DNA (□—□) and unlabelled NPV-DNA (○—○), respectively. Further details on the hybridization conditions and hydroxylapatite chromatography are indicated in Table 6. fss = single-stranded fraction.

Demonstration of NPV sequences in samples of the B. brassicae host DNA

The number of *B. brassicae* NPV copies integrated to some extent with its host DNA was determined by following the kinetics of reassociation of ^{32}P--labelled NPV-DNA with unlabelled host DNA, isolated from healthy looking

Table 7. Reassociation kinetics of the *B. brassicae* NPV-DNA in the presence of the *B. brassicae* host DNA

Time of sampling	^{32}P-lab. NPV-DNA and unlab. host DNA of larvae with latent virus inf.		^{32}P-lab. NPV-DNA and unlab. host DNA of healthy larvae		^{32}P-lab. NPV-DNA and unlabelled NPV-DNA	
in hr	cpm in fss	$^{1}/fss$	cpm in fss	$^{1}/fss$	cpm in fss	$^{1}/fss$
0	850	1.000	946	1.000	850	1.000
1	821	1.035	944	1.002	765	1.111
2	786	1.080	946	1.000	738	1.150
4	720	1.180	946	1.000	629	1.350
6	680	1.250	944	1.002	586	1.450
12	556	1.525	944	1.002	428	1.995
22	447	1.900	941	1.005	-	-

t = time of sampling in hr, cpm = counts per min, $^{1}/fss$ = ^{1}/single-stranded fraction.
The reaction mixture of NPV-DNA with host DNA isolated from larvae suggesting a latent NPV infection (from Hungary) contained 1.24×10^{-3} µg (854 cpm) of ^{32}P-labelled NPV-DNA and 160 µg of unlabelled host DNA. Negative control contained 1.38×10^{-3} µg (946 cpm) of ^{32}P-labelled NPV-DNA with 160 µg of unlabelled host DNA isolated from healthy larvae (collected in an allotment). Positive control contained 1.24×10^{-3} µg (854 cpm) of ^{32}P-labelled NPV-DNA with 0.39 µg of unlabelled NPV-DNA. The hybridization and the analysis of the single-stranded fraction are indicated in Materials and Methods.

4th instar larvae in which we demonstrated a latent virus infection (Table 5). The insects originated in Hungary. The reaction mixture for the study of viral sequences in host DNA contained 1.24 x 10^{-3} µg of ^{32}P-labelled NPV-DNA and 160 µg of host DNA isolated from larvae reared from surface sterilized eggs. The positive control contained 1.24 x 10^{-3} µg of ^{32}P-labelled NPV-DNA and 0.39 µg of unlabelled NPV-DNA which represented the reassociation rate of about 4.5 virus genomes per diploid insect cell in 160 µg of host DNA. The negative control was a mixture of 1.38 x 10^{-3} µg of ^{32}P-labelled NPV-DNA and 160 µg of unlabelled host DNA extracted from *B. brassicae* healthy caterpillars. These caterpillars originated from eggs which were collected in an allotment in Wageningen in 1975. The data of the reassociation kinetics are given in Table 7 and depicted in Fig. 2. As seen from Fig. 2, the reassociation rate of ^{32}P-labelled NPV-DNA in the presence of host DNA extracted from larvae with a latent infection, was

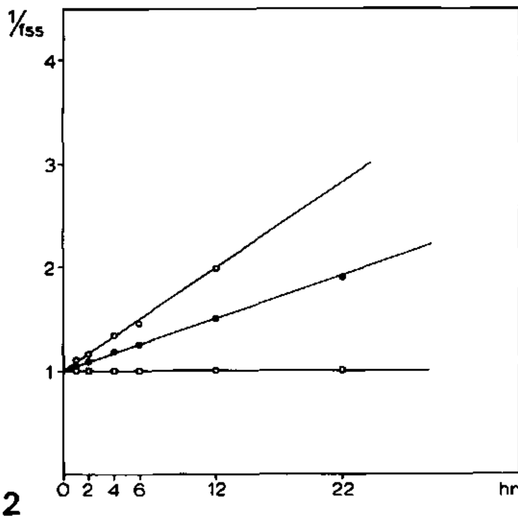

Fig. 2. Reassociation of ^{32}P-labelled *B. brassicae* NPV-DNA in the presence of *B. brassicae* host DNA from latent infected larvae (●—●), *B. brassicae* host DNA from healthy caterpillars (□—□) and unlabelled NPV-DNA (○—○), respectively. Further details on the hybridization conditions and hydroxylapatite chromatography are indicated in Table 7. fss = single-stranded fraction.

about half that of the positive control. This means that about 2.5 of viral copies were present per diploid quantity of host DNA. This result was also confirmed by calculating the viral equivalents per diploid quantity of host DNA with the equation of Sharp et al. (1974). The mol wt of host DNA was taken 1.68 x 10^{11} and the mol wt of virus genome 8.9 x 10^{7}.

DISCUSSION

The results of light microscopy shown in Table 1, indicate that the number of healthy carriers was reduced by surface sterilization of eggs to less than 1/3 the number of carriers reared from untreated eggs. From this it can be concluded that the virus can be transmitted to the progeny on the surface of eggs. The most efficient method of dispersal is probably the flight or migration of adults as studied by Tanada (1964). Alternatively transovum transmission of NPV can be through contamination in female genitalia as described by Martignoni and Milstead (1962). In both examples of this transmission, the virus occurs on the outside of the egg so that contamination can be prevented by surface sterilization of eggs. Because the polyhedra were also found in smears of fat-cells of larvae reared from surface sterilized eggs, it can be concluded that virus is also transmitted inside eggs. The biological tests show that extracts prepared from various development stages of insects were infective (Table 3). From this observation it may be concluded that generation-to-generation transmission is followed by transstadial transmission of virus material. After generation-to--generation transmission, the virus in insects may either multiply abundantly, slightly or not at all. Therefore, although some carriers die during their development, most of them develop into normal adults under favourable rearing conditions (Tables 2 and 4). In this way it is again possible to transmit the virus through the egg. Our observations on polyhedra in fat cells of healthy insects confirm the results of Roegner and Aust (1949) who found polyhedra in stocks of *B. mori* larvae which had shown no symptoms of virus infection for several years. However the polyhedra that we observed in fat cells of healthy insects represented only one particular form of a latent virus infection in insects. The presence of other forms of latent virus infection were indicated by experiments on the activation of latent virus infections and accidentally when larvae were transferred from an old dry diet to a freshly prepared one. Also, when temperature-humidity regulation failed, more larvae showed symptoms of nuclear polyhedra disease than were to be expected from the microscopic observations of fat cells. The presence of a latent virus infection in a form other than that of polyhedra found, was indicated by reassociation kinetics of NPV-DNA with host DNA. The *A. orana* host DNA was isolated from strongly selected 4th instar larvae which were checked for the presence of polyhedra in their fat cells. Larvae containing polyhedra (about 3%) were not used for preparation of host DNA. The likelihood that host DNA was still contaminated by DNA originating

from polyhedra already formed was lessened by purifying host DNA in *p*H range 7 - 8.6 and the purification procedure was accomplished by CsCl gradient centrifugation. The results of reassociation kinetics analysis suggest the presence of a small amount of NPV-DNA which can be integrated to some extent with that of the host DNA. It is not known whether this amount represents a complete virus genome in each cell of 3% of the larvae or whether a part of the genome is in each cell of each larvae. Our results also did not show whether the virus DNA in *A. orana* larvae is covalently closed, linearly integrated with alkali labile bonding or whether there is another unknown association of virus DNA with host DNA.

The *B. brassicae* larvae were mass reared in preserving jars because they are too big for rearing in tubes. Accordingly, larvae were reared only from surface sterilized eggs. During the rearing of larvae (having a latent virus infection) no horizontal infection was detected. Our experiences indicate that the faeces of larvae carrying a latent virus infection are not a source of infection. Most probably the latent virus infection in the form of polyhedra is located only in fat cells and from here it cannot be liberated.

Infectivity experiments on extracts prepared from various developmental stages of *B. brassicae* (Tables 4 and 5) showed that, as in *A. orana*, generation-to--generation transmission of virus is followed by transstadial transmission and that in some latent infected insects, virus replicate. The *B. brassicae* larvae with a latent virus infection were obtained from Dr. Varjas (Budapest) who reared these insects for some years from repeatedly surface sterilized eggs without symptoms of a polyhedral disease. The rearing was continued in our laboratory for 10 generations. The eggs were surface sterilized with 10% formaldehyde for 60 min. Prolongation of the duration of this treatment up to 120 min as well as other treatments such as with sodium hypochloride had no effect on the reduction of the number of NPV carriers. The fat cells of some healthy larvae still contained polyhedra and these polyhedra were only one form of a latent virus infection of these insects. Another form of latent virus infection was detected by the reassociation kinetics of ^{32}P-labelled NPV-DNA with host DNA prepared from rigorously selected 4th instar larvae with a latent virus infection. The comparison of the reassociation rate of ^{32}P-labelled viral DNA in the presence of host DNA prepared from larvae with a latent viral infection with the reassociation rate of positive and negative control shows that there were about 2.5 copies of viral genome per diploid quantity of host DNA. Because no polyhedra were seen in inocula prepared from eggs of both insects and these inocula were not infective, we think that the virus material transmitted through the eggs

might be in a non infective state, probably DNA, which could be associated with host DNA.

Until now only the existence of transovarial transmission of virus in general has been discussed in literature. The form of the virus for transovarial transmission has not yet been studied. Results obtained suggest that the virus DNA could be integrated to some extent with that of the host DNA (thus the virus could be associated with host DNA in the stage of an egg too). However, the results do not exclude another way of transovarial transmission of virus, probably in another form than virus DNA, perhaps by seminal fluids. The virus or polyhedra originating from contaminated male or female could be introduced in the egg cell through the micropyle by seminal fluids. At present it is not known what happens from the moment of contamination until the polyhedra can be observed in fat cells of larvae. Possibly the hatching larvae consume this virus with content of eggs and the virus penetrates the gut epithelium and multiplies in the cells of blood or tissues as described by Day et al. (1958). Probably when the initial dose is small, the virus governs the latency.

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GENERAL DISCUSSION AND SUMMARY

In infectivity experiments some *A. orana* larvae died after being inoculated with an inoculum containing NPV isolated from *B. brassicae*. The polyhedra formed upon infection occluded single virus particles, whereas the inoculum contained polyhedra with bundles of virus particles. This change could be explained either by activation of a virus in *A. orana*, which is singly embedded, or the inoculum from *B. brassicae* had infected *A. orana* and consequently the inclusion of virus particles in outer membranes is controlled by the hosts. This thesis describes studies performed to discriminate between both possibilities. Therefore, the first task was to characterize the virus particles from *B. brassicae* and *A. orana* NPV and their polyhedra by different techniques (Chapter 1).

The properties of the NPV of *A. orana* and of *B. brassicae* as observed with the electron microscope and polyacrylamide gel electrophoresis are similar to those found for many other NPVs. The polyhedra of both NPVs differ in size and shape. Most of the *A. orana* polyhedra are globular and range in diameter from 1-2 μm. Most of the *B. brassicae* polyhedra are hexagonal or pentagonal in outline and range in diameter from 1.5-4 μm. Analysis of polyhedral protein by polyacrylamide gel electrophoresis shows the presence of two polypeptides of molecular weight 28,000 and 54,000 Daltons.

Treatment of the polyhedra of both viruses with sodium carbonate ruptures the polyhedral membrane and the virus particles and polyhedral proteins are released. The virus particles of *A. orana* polyhedra are singly embedded in the polyhedral matrix and have a size of 250 x 60 nm. The multiply embedded virus particles of *B. brassicae* have a size of 347 x 113 nm. Analysis of the viral proteins by SDS-polyacrylamide gel electrophoresis showed that NPV of *A. orana* has 5 polypeptides of 68,000, 48,000, 39,000, 32-34,000, and 28,000 Daltons, respectively. Those of the NPV of *B. brassicae* were 69,000, 57,000, 46,000, 34-39,000, and 28,000 Daltons, respectively.

In the polyhedral membrane fractions of both polyhedra one polypeptide of molecular weight of 28,000 Daltons as estimated by polyacrylamide gel electrophoresis, was found.

Due to proteolytic activity associated with the polyhedra, which is evident after dissociation of the polyhedra, it was difficult to establish the number of polyhedral proteins and their molecular weight (Chapter 2). The electrophoretic pattern of polyhedral proteins of *A. orana* and *B. brassicae* polyhedra dissociated in alkali differed from those proteins obtained by other means. Six to seven polypeptides with molecular weights between 28,000 and 8,000 Daltons were found after incubation at *p*H 10.5. After inactivation of the enzyme only two polypeptides with molecular weights of 28,000 and 26,000 Daltons were observed. When the polyhedral proteins were analysed without incubation at *p*H 10.5 also two proteins were found, but their molecular weight was 54,000 and 28,000 Daltons.

On the basis of the results described in Chapters 1 and 2 it can be concluded that the virus particles of *B. brassicae* and *A. orana* NPV differ with respect to size, the way of occlusion, and the form and size of the polyhedra involved. Protein analysis by polyacrylamide gel electrophoresis reveal some difference in molecular weight of viral protein but no significant difference in the protein composition of their polyhedra. Further analyses of amino acid composition and sequence of these proteins is necessary to elucidate possible differences.

To differentiate further between both viruses their genomes were analysed (Chapter 3). Both genomes are circular double-stranded DNA molecules. The molecular weights of *A. orana* and of *B. brassicae* NPV-DNAs are 6.7×10^7 and 8.9×10^7 Daltons, respectively as determined by electron microscopy and by renaturation kinetic analysis. The renaturation also indicated that both genomes contain only unique sequences. The buoyant density in CsCl of the NPV-DNA of *A. orana* and of *B. brassicae* is 1.694 and 1.696 g/cm^3, respectively. These values are in good agreement with (G+C) contents of 34.5 and 37%, respectively as determined by thermal denaturation. The digestion of the *A. orana* and of the *B. brassicae* NPV-DNA with endonuclease *Eco* RI resulted in completely different electrophoretic patterns. Also in experiments on competition hybridization no homology between these genomes was found. The conclusion of these studies is that these two NPVs can be clearly differentiated by their DNA properties.

In order to study the occurrence of viral DNA in uninfected larvae the DNA of *A. orana* and *B. brassicae* was isolated and the complexity studied (Chapter 4). The genomes of *A. orana* and of *B. brassicae* differ in their kinetic complexity as estimated from the reassociation data on hyperchromicity, but they are both relatively small and show remarkable similarity in the extent of intragenome homology. A haploid cell of *A. orana* has a DNA equivalent of 4.2×10^{10} and that of *B. brassicae* of 8.4×10^{10} Daltons. The intragenome homology was estimated to be 10 and 9% for *A. orana* and *B. brassicae* genome, respectively. The (G+C) content, estimated by thermal denaturation, was found to be 36.2% for the *A. orana* genome and 35.8% for the *B. brassicae* genome.

The results obtained during rearing of insects from surface-sterilized eggs and from untreated eggs showed that the NPV of *A. orana* and of *B. brassicae* can be transmitted to the progeny of these insects on the outside of the eggs (transovum) as well as inside the eggs (transovarially) (Chapter 5). Evidence for transovarial transmission was also obtained from reassociation of viral DNA with the host DNA of homologous insects reared from surface-sterilized eggs. These experiments revealed the presence of viral sequences in host DNA: 0.03 and about 2.5 viral copies for the diploid quantity of the *A. orana* and of the *B. brassicae* host DNA, respectively.
Results obtained in infectivity experiments with insects in various developmental stages showed that transstadial transmission is a prerequisite for generation-to-generation transmission.

The presence of a latent virus infection in both insects could also be demonstrated in cross-inoculation experiments (Chapter 6). When the larvae of *A. orana* and of *B. brassicae* were inoculated with polyhedra of the reciprocal species, the number of larvae containing polyhedra increased compared with that of the control. Comparison of the restriction endonuclease *Eco* RI pattern of DNA isolated from polyhedra used as inocula with that from polyhedra obtained after cross-inoculation indicated that both viruses are not cross-infective but that they activate a latent virus infection in both insects. Because the cross-inoculation experiments were done under laboratory conditions (as aseptic as possible), it could be concluded that the *B. brassicae* NPV is not suitable for biological control of *A. orana* in the field, because this virus is not cross-infective.

ALGEMENE DISCUSSIE EN SAMENVATTING

Uit waarnemingen die met de elektronenmicroscoop werden gedaan en uit de resultaten die na analyse in polyacrylamidegels werden verkregen, blijkt dat de kernpolyedervirusses (NPVs) van *A. orana* en *B. brassicae* in grote lijnen dezelfde eigenschappen hebben als NPVs van andere Lepidopteren. De polyeders van *A. orana* en *B. brassicae* verschillen evenwel aanzienlijk in grootte en vorm. De meeste polyeders van *A. orana*-NPV zijn rond en hebben een diameter van 1-2 μ, terwijl de meeste polyeders van *B. brassicae*-NPV een hexagonale of pentagonale omtrek laten zien met een diameter van 1.5-4 μ. Het eiwit uit polyeders dat zonder incubatie in alkalische omstandigheden met polyacrylamidegelelektroforese werd onderzocht, bleek uit twee polypeptiden van ongeveer 28.000 en 54.000 Daltons te bestaan. Wanneer men de polyeders van beide virussen met natriumcarbonaat behandelt, vallen ze uit elkaar en komen de virusdeeltjes, de polyedereiwitten en de polyedermembranen vrij. Het kernpolyedervirus van *A. orana* verschilt qua afmetingen geheel van dat van *B. brassicae*. De virusstaafjes van *A. orana*-NPV zijn 250 nm lang, en hebben een diameter van 60 nm. Ze zijn afzonderlijk ingebed in het polyedereiwitkristal. De staafvormige virusdeeltjes (347 x 113 nm) van *B. brassicae*-NPV worden na door een membraan in groepjes te zijn gebundeld, in polyeders opgenomen.

Analyse van *A. orana*-NPV-eiwitten op polyacrylamidegels resulteerde in detectie van vijf polypeptiden met een molecuulgewicht van 68.000, 48.000, 39.000, 32-34.000 en 28.000 Daltons. Er werden eveneens vijf polypeptiden met molecuulgewichten van 69.000, 57.000, 46.000, 34-39.000 en 28.000 Daltons gevonden wanneer de deeltjes van *B. brassicae*-NPV werden geanalyseerd. Door incubatie van de polyeders in alkalisch milieu (*p*H 10.5) verandert het patroon van de polypeptiden op polyacrylamidegels. Er worden dan 6-7 polypeptiden met molecuulgewichten tussen 8.000 en 28.000 gevonden. Na inactivatie van de proteolytische enzymen, die met de polyeders zijn geassocieerd en die in een alkalisch milieu worden geactiveerd, worden er slechts twee polypeptiden gevonden. Hun molecuulgewicht is 28.000 en 26.000 Daltons.

Het genetische materiaal van beide virussen is een circulair dubbelstrengig DNA molecuul. Het molecuulgewicht van het *A. orana*-NPV-DNA is 6.7 x 10^7 Daltons, en dat van het *B. brassicae*-NPV-DNA is 8.9 x 10^7 Daltons. De molecuulgewichten werden berekend aan de hand van de resultaten die uit elektronenmicroscopische lengtemetingen waren verkregen, alsmede uit hybridisatie-experimenten. De laatste experimenten toonden aan dat beide genomen alleen unieke base-volgorden bezaten. De zweefdichtheden van het *A. orana*- en *B. Brassicae*-NPV-DNA waren respectievelijk 1.694 en 1.696 g per cm^3. Deze waarden komen overeen met een G+C gehalte van 34.5 en 37.1%, zoals die in smelttemperatuur (Tm)-studies werden gevonden. Na behandeling van het DNA met het restrictie-enzym *Eco* RI bleken beide NPV-DNA-moleculen totaal verschillende elektroforetische patronen te geven. Ook in competitieve hybridisatie-experimenten kon geen homologie tussen de genomen van deze virussen worden aangetoond.

Het gastheergenoom van *A. orana* verschilt van dat van *B. brassicae* in kinetische complexiteit, zoals dat uit de reassociatie-snelheden kon worden vastgesteld. Het haploide genoom van *A. orana* is 4.2 x 10^{10} Daltons en dat van *B. brassicae* is twee maal zo groot, namelijk 8.4 x 10^{10} Daltons. Beide zijn betrekkelijk klein en bezitten ongeveer 10% repetitieve sequenties. Het G+C gehalte van het *A. orana*- en *B. brassicae*-genoom bleek zoals dat uit de Tm waarden kon worden berekend, 36.2 en 35.8% te zijn.

In een aantal experimenten werd nagegaan op welke wijze de NPVs van respectievelijk *A. orana* en van *B. brassicae* op de nakomelingschap kan worden overgedragen. Daartoe werden eitjes van vlinders die uit eitjes zonder voorafgaande ontsmetting van het oppervlak waren opgekweekt, in twee even grote groepen verdeeld. Het oppervlak van de eitjes van de ene groep werd wel en dat van de andere groep werd niet ontsmet. Van beide groepen stierven larven ten gevolge van polyederziekte. Van de larven die afkomstig waren van de behandelde eitjes stierven er relatief minder dan van uit de groep die onbehandeld waren gebleven. Hieruit kan geconcludeerd worden dat de NPVs van *A. orana* en *B. brassicae* vía het ei op de nakomelingschap kunnen worden overgedragen. De resultaten duiden er op dat het virus zowel in het ei als aan de buitenkant kan voorkomen. Deze conclusie wordt ondersteund door resultaten uit hybridisatie-experimenten tussen het virus-DNA en het gastheer-DNA. Uit de reassociatie-snelheden kon worden berekend, dat in het hybridisatie-mengsel 0.03 en 2.5 kopieën van het *A. orana* en *B. brassicae* NPV-genoom per diploïde gastheercel aanwezig waren. Er is niet vastgesteld of het virus-DNA al of niet covalent gebonden aan het gastheer-DNA voorkomt.

In experimenten waarin de larven van *A. orana* en van *B. brassicae* geïnoculeerd waren met polyeders van de reciproke species, was het aantal larven dat polyeders bevatte groter dan dat in de ongeïnoculeerde controlegroep. Het DNA uit de polyeders waarmee werd geïnoculeerd, en die tijdens de infecties waren gevormd, werd gefragmenteerd met het restrictie-enzym *Eco* RI. Het DNA uit polyeders die tijdens de infectie waren gevormd bleek te verschillen van het DNA uit de polyeders waarmee de larven geïnfecteerd waren. Het DNA, dat na de infectie uit de polyeders werd geïsoleerd, was nl. identiek met het DNA dat uit het soort-specifieke virus werd geïsoleerd. Uit deze resultaten kan geconcludeerd worden dat geen van beide virussen tot "cross-infection" in staat is. Dit betekent voorts dat het NPV van *B. brassicae* niet gebruikt kan worden in de biologische bestrijding van *A. orana*.

Z obsahu práce

Vo všeobecnosti možno povedať že táto práca popisuje charakteristiku dvoch vírusov z ktorých jeden sa rozmnožuje (alebo je latentne prítomný) v *Adoxophyes orana* larvách a druhý v *Barathra brassicae* larvách.

Kapitola prvá: Popisuje štruktúru a chemické zloženie týchto vírusov. Oba vírusy sú tyčinky. *A. orana* vírus má rozmery 250 x 60 nm a každá tyčinka má dva obaly: vnútorný a vonkajší. *B. brassicae* vírus má rozmery 347 x 113 nm a každá tyčinka má len zvláštny vnútorný obal. Vo vonkajšom obale možno najísť 2-8 tyčiniek. Tyčinky oboch vírusov sú umiestnené v bielkovnových kryštaloch. Rozmery týchto kryštalov sú 1-4 μm (Obr. 1). Elektroforéza ukázala že oba vírusy sa skladajú z piatich rozličných bielkovín. Kryštal týchto vírusov sa skladá len z dvoch bielkovín.

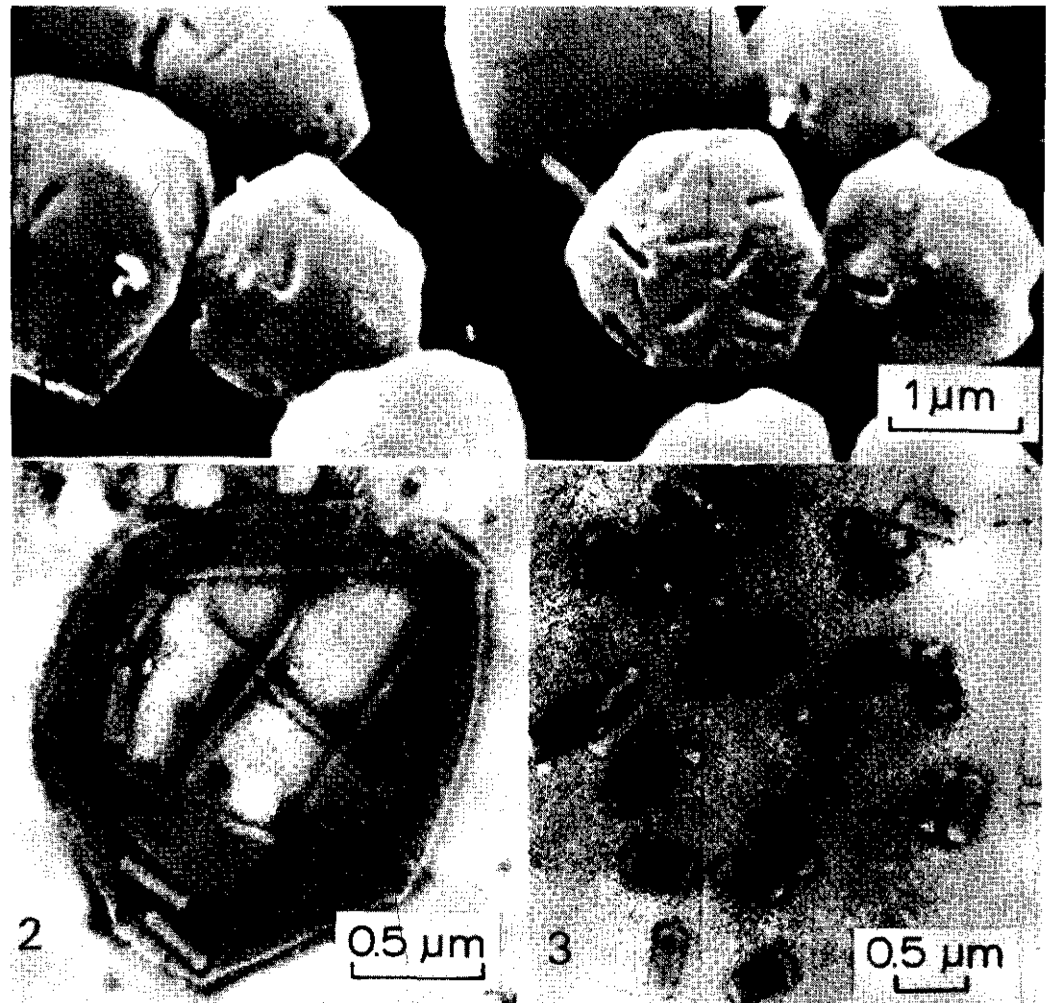
Kapitola druhá: V čase inkubovania proteinových kryštalov v alkalickom prostredí tieto sa rozpadajú a vírusové tyčinky sa oslobodia z týchto kryštalov a ich blán. Blana čiastočne rozpadnutého kryštalu je vidieť na Obr. 2. Vírusové tyčinky v čase keď ešte nie su oslobodené z bielkovín kryštalu sú vidieť na Obr. 3. Rozpad kryštalu je pravdepodobne spôsobený enzymami ktoré sa nachádzajú na vonkajšej strane kryštalu. Tieto enzymy nie su vírusového pôvodu. Aktívna činnosť týchto enzymov sa da znížit ohriatim.

Kapitola tretia: Každý vírus obsahuje jednu molekulu deoxyribonucleovej kyseliny (DNK). DNK oboch vírusov je uzavretá kruhová molekula. *A. orana* vírus obsahuje DNK molekulu ktorej veľkosť je 6.7×10^7 Daltonov a *B. brassicae* vírus obsahuje DNK molekulu ktorej veľkosť je 8.9×10^7 Daltonov. Príklad jednej DNK molekule je vidieť na Obr. 4. Hybridizačné pokusy s nucleovými kyselinami oboch vírusov ukázali ze oba vírusy sú úplne rozličné. Žiadna identita medzi obomi vírusami sa nepoukázala ani v prípade keď ich DNK boli rozložené enzymaticky a produkty enzymatickej reakcie boli analyzované elektroforeticky.

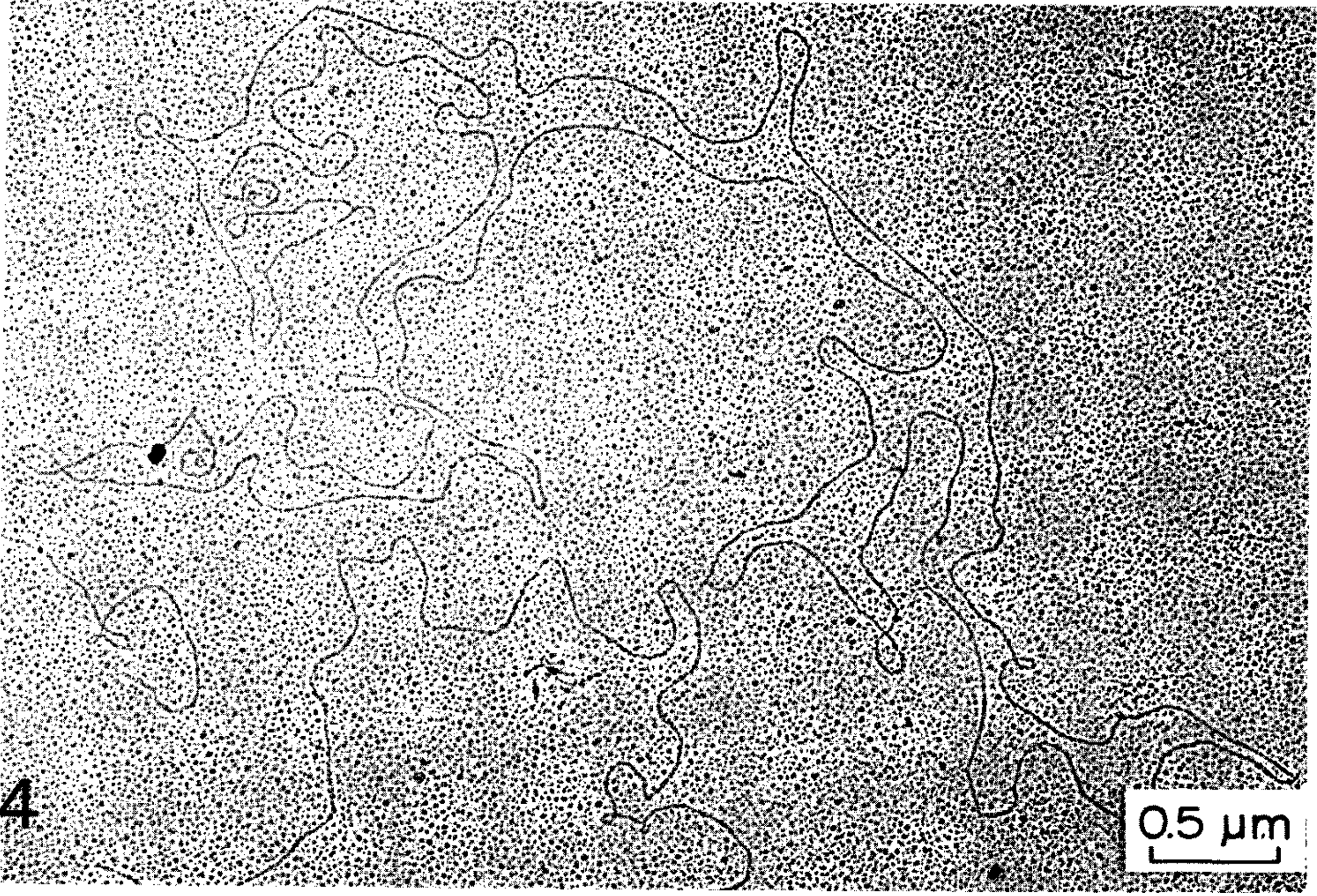
Kapitola štvrtá: táto kapitola popisuje charakteristiku deoxyribonucleovej kyseliny (chromozómov) oboch lariev. Molekulárna váha *A. orana* haploidného genómu bola ustanovená 4.2×10^{10} Daltonov a molekulárna vaha *B. brassicae* genómu 8.4×10^{10} Daltonov. Táto kapitola ďalej popisuje vnútornú stavbu týchto nucleových kyselín a ich percentuálne množstvo guaninu a cytosinu.

Kapitola piata: Účelom tohoto štúdia bolo poukázať jestli *B. brassicae* vírus sa môže rozmnožovať v *A.orana* larvách. Pred niekoľkými rokmi bolo poukázané že keď *A. orana* larvy boli infikované s *B. brassicae* vírusom, vírus obsiahnutý po infikovaní mal iné vlastnosti ako vírus ktorý bol použitý pre infikovanie (viď kapitolu prvú). Zmena mohla nastať dvomi spôsobami: vírus z inocula sa zmenil pod vplyvom hosťa alebo cudzí vírus aktivoval latentný vírus v *A. orana* larvách. Práca poukázala ze druhá možnosť bola pravdou. Skutočný dôkaz poskytla analýza nucleových kyselín oboch vírusov za pomoci elektroforézy.

Kapitola šiesta: Že v oboch druhoch škodcov vírus latentne prítomny bol, bolo poukázane pomocou hybridizačnych pokusov. Biologickými pokusami bolo poukázané že oba vírusy sú prenášane z pokolenia na pokolenie na vonkajšej ako i vnútornej strane vajíčka.



Obr. 1. Bielkovinový kryštal v ktorom sa nachádzajú vírusové tyčinky.
Obr. 2. Blana kryštalu. Obr. 3. Vírusové tyčinky v čiastočne rozpadnutom kryštale.



Obr. 4. Príklad nucleovej kyseliny ktorá je uzavretá vo vírusovej tyčinke.

CURRICULUM VITAE

Mária Jurkovíčová werd op 20 mei 1942 geboren te Veľké Leváre (Tsecho-Slowakije). In 1960 behaalde zij het diploma van de 'stredná škola' (instelling voor voorbereidend wetenschappelijk onderwijs) te Sološnica en in 1966 het ingenieursdiploma (studierichting: tuinbouw) aan de Landbouwhogeschool te Brno. Gedurende de jaren 1961-1966 was de auteur tevens docente aan de middelbare landbouwschool te Cerová-Lieskové. Na enkele jaren praktijkervaring begon zij in 1970 haar studie aan de Landbouwhogeschool te Wageningen welke in 1973 werd afgerond met het behalen van het ingenieursdiploma in de studierichting planteziektenkunde (hoofdvak virologie, verzwaard bijvak microbiologie en bijvak erfelijkheidsleer). Gedurende de jaren 1970-1972 was de auteur tevens werkzaam op de afdeling Microbiologie van de eerder genoemde hogeschool. Vanaf september 1973 tot september 1977 was zij als wetenschappelijk assistente verbonden aan de afdeling Virologie van de Landbouwhogeschool te Wageningen. In het studiejaar 1977-1978 was zij als docente biologie verbonden aan de Stichting tot opleiding van analisten te Wageningen. Thans is zij werkzaam als wetenschappelijk medewerkster op de afdeling Milieu-onderzoek van de N.V. tot Keuren van Elektrotechnische Materialen te Arnhem.