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A NUCLEAR-POLYHEDROSIS VIRUS FROM HELIOTHIS
PUNCTIGERA WALLENGREN
(LEPIDOPTERA:NOCTUIDAE)

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SUMMARY

A nuclear-polyhedrosis virus from an Australian species of *Heliothis*, *H. punctigera* Wallengr., is described on the basis of the morphology and sizes of the polyhedra and virions, its pathology, and incubation periods in laboratory infections.

The virus could be distinguished serologically from viruses from *Anthela varia* Walk., *Orgyia anartoides* (Walk.), *O. australis* Walk. and *Pterolocera amplicornis* Walk. by means of agar gel diffusion tests using the polyhedral protein, but not from a virus from *H. armigera* (Hubn.). The viruses from *H. punctigera* and *H. armigera* showed reciprocal cross-infectivity, but the *H. punctigera* virus was not infective for six species from other genera.

I. INTRODUCTION

Nuclear-polyhedrosis has been recorded in five species of *Heliothis*, namely *H. armigera* (Hubn.) (= *H. obtectus*), *H. peltigera* Schiff., *H. phloxiphaga* Grote and Robinson, *H. virescens* (F.) and *H. zea* (Boddie) (Ignoffo 1966c), and a commercially produced preparation of nuclear-polyhedrosis virus from *H. zea* has been registered for use on cotton in the United States (Federal Register 35 (230) Dec. 9, 1970). In Australia four species of *Heliothis* are known to occur, *H. armigera*, *H. assulta* Guen., *H. punctigera* and *H. rubescens* (Walk.) (Common 1953). *H. punctigera* has been recorded only from Australia and Cocos I. (Common 1953) but in Australia it is the most common and the most serious pest of the four. It is a major pest of cotton, maize, tobacco, linseed and such small crops as tomatoes and cut flowers. On lucerne (*Medicago sativa* L.) large populations of *Heliothis* larvae, predominantly *H. punctigera*, are frequently destroyed by disease in southern and central Queensland in September and October (spring), and sampling has indicated that nuclear-polyhedrosis is the main cause of death. Studies of the virus obtained were initiated to investigate its possible use as a partial substitute for chemical insecticides. This paper contains a description of the morphology, some serological characteristics, the pathology and the pathogenicity of the virus.

II. MATERIALS AND METHODS

Virus.—The virus in the present study was obtained from infected larvae and the progeny of moths from three centres in Queensland, namely Warwick, Beaudesert and Biloela, between January 1967 and October 1968. Virus epizootics occurred on lucerne at Beaudesert in October 1967 and 1968 and at Biloela in October 1968.

Suspensions of polyhedra from triturated larvae were initially purified by filtration through two layers of cheesecloth and subsequently by low-speed and high-speed centrifugation and stored, frozen, in distilled water at -20°C . Polyhedra for infectivity tests were suspended in 0.2% 'Teepol' wetting agent to which 2 000 units each of penicillin and streptomycin per ml were sometimes added to control bacterial infections. Counts of polyhedra were done with a Hawksley-Helber counting chamber (Thoma ruling; depth 0.01mm) using phase contrast at a magnification of 225X.

Larvae.—The larvae were the progeny of moths collected over lucerne at Beaudesert. They were reared on bean pods (*Phaseolus vulgaris* L.) washed with 70% ethanol (Kirkpatrick 1962). Batches of approximately 10 were reared in 10.5 cm diam. x 15 cm high wide-neck glass jars, and individual larvae were confined in a 5 cm diam. x 7 cm high glass jar inverted on filter paper in half a petri dish.

Infection of larvae.—Larvae were infected by being allowed to feed on 2.5 cm lengths of bean slit down one side with the virus dosage in the depression under the seed. This site was chosen owing to the tendency of early instar larvae to feed on the underside of seeds. Eggs were contaminated by dipping in a suspension of polyhedra in 0.2% Teepol containing a small quantity of fresh haemolymph as a sticker.

To investigate the effect of larval age (instar) on incubation period, larvae of each instar were fed 5×10^8 polyhedra. Dosages containing 10^2 to 10^8 polyhedra, prepared from 10-fold dilutions of a stock suspension, were used to determine the effect of virus dosage on the incubation period in fifth instar larvae. Larvae which did not consume the dosage or which moulted within 2 days were discarded. This was necessary owing to the marked tolerance to infection shown by final instar larvae.

Histopathology.—Fifth instar larvae were used in histopathological studies. Dosed larvae were sampled daily in pairs, one being killed with chloroform and the other with an injection of Bouin-Dubosq-Brasil fixative. The blood and tissues from the former larva were examined immediately by phase contrast. The other larvae was fixed for sectioning and staining by the method of Hamm (1966).

Cytopathology.—For cytopathological studies fourth and fifth instar larvae reared on an artificial diet based on that of Shorey and Hale (1965) were dosed with 5×10^8 polyhedra in a 1 cm³ cube of diet from which formaldehyde had been omitted. Treated and untreated larvae were sampled at 1, 2, 3 and 5 days. Small pieces of midgut and fat body or trachea were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2, post-fixed in buffered osmium tetroxide (Millonig 1962) and embedded in 'Araldite' for sectioning. Sections cut with an LKB Ultratome were double-stained with uranyl acetate and lead citrate (Reynolds 1963) and examined with a Siemens Elmiskop 101 electron microscope.

Virus descriptions.—A Siemens Elmiskop 1A electron microscope was used to determine the morphology of the polyhedra and virions. The size and shape of the polyhedra were determined from unstained preparations. For sectioning, the polyhedra were fixed in glutaraldehyde and embedded in Epon. Sections were cut and stained as for the cytopathological studies.

Polyhedra were dissolved in 0.005 M Na_2CO_3 for 30–60 min following the method of Bergold (1963) and the liberated virions negatively stained with 1% or 2% potassium phosphotungstate at pH 7.0. The liberated virions were fragile and very difficult to obtain intact after removal of the outer (developmental) membrane. A number of modifications to overcome the tendency to rupture and flatten were unsuccessful. These included short-period degradation of the polyhedra in which the reaction was stopped by neutralization with 0.1 N HCl, fixation with 3% glutaraldehyde at pH 7.3 after degradation, and the use of 1% uranyl formate, 1% uranyl acetate and phosphotungstate pH 5.0 as negative stains.

Serology.—The serological relationships between the polyhedral proteins of viruses were investigated using the Ouchterlony agar gel double diffusion technique (Crowle 1961, pp. 69-75).

Antigens.—These were approximately saturated solutions of purified polyhedra in dilute Na_2CO_3 containing 0.05 M NaCl. A concentration of 0.005 M Na_2CO_3 was used for viruses from *H. armigera*, *H. punctigera*, *O. anartoides* and *O. australis*, and 0.015 M Na_2CO_3 was used for the viruses from *A. varia* and *P. amplicornis*.

Sodium azide to a final concentration of 0.5% was added to all antigens as a preservative (Koenig and Jankulowa 1968), and the antigens were stored in the refrigerator at 5°C. Tests were carried out with antigens normally within several weeks of their preparation, as components of some virus antigens were lost during prolonged storage. This was indicated by the fact that antisera to fresh preparations of these viruses showed greater reactivity with some antigens prepared from other viruses.

To prepare virus antigens from individual *H. punctigera* larvae with polyhedrosis, the larvae were macerated in distilled water and centrifuged at 1 000 g for 5 min, and the sediment was washed with distilled water. The sediments were suspended in 0.25 ml 0.005 M Na_2CO_3 —0.05 M NaCl, and 0.06 M Na_2CO_3 was added dropwise until much of the polyhedra had dissolved. The extracts were held overnight in the refrigerator (5°C), during which time the polyhedra dissolved. The solutions obtained after centrifugation at 4 000 g for 5 min were used to test for serological specificity.

Control antigens were prepared by grinding 1 g of frozen healthy larvae in 10 ml of alkali of the concentration corresponding to that used for dissolving the polyhedra and clarifying by centrifugation at 4 000 g for 5 min.

Antisera.—Three large male guinea pigs were used to prepare the antiserum to the *H. punctigera* virus. Each received an intraperitoneal injection of 3 mg antigen protein, estimated by the method of Waddell (1956), followed by 1.5 mg antigen protein emulsified in complete Freund's adjuvant. The guinea pigs were bled 3 weeks later and the sera were stored at -20°C without added preservative.

Agar gel diffusion tests.—These were run in 9 cm diam. petri dishes in 12 ml 0.85% 'Difco' Special Agar-Noble containing 0.85% sodium chloride and 0.5% sodium azide as employed by Koenig and Jankulowa (1968), and

buffered at pH 8.6 with veronal with an ionic strength of 0.1. The diameter of the reactant wells was 7 mm, the distance between the wells was 4 mm, and 0.025 ml volumes of the reagents were used. Undiluted antigens were used in the outer wells and the concentration of the antiserum in the central well was adjusted to give, in most instances, a single well-defined precipitin line with its corresponding antigen. Veronal buffer pH 8.6, ionic strength 0.1, was used as a diluent to avoid possible precipitation of the polyhedral protein, which occurred at neutrality or a lower pH. The tests were done at 37°C overnight in a moist chamber.

III. RESULTS AND DISCUSSION

(a) Description of Virus

The polyhedra were irregular in shape and contained the virions embedded singly and at random within. The sizes of the polyhedra and virions are given in Table 1 and compared with those from *H. armigera* and *H. zea* from available data in Table 2. Data concerning the sizes of polyhedra and virions from *H. peltigera*, *H. phloxiphaga* and *H. virescens* were not available. Formal statistical comparisons with the sizes of the particles from *H. armigera* reported by Bergold and Ripper (1957) are not possible as the numbers of measurements were not recorded by those authors and it was not clear whether standard deviations or standard errors were given. The lengths of the virions from *H. zea* produced by Na₂CO₃ treatment were significantly larger than those from *H. punctigera* ($P < 0.001$). However, it appears unlikely that these viruses could be differentiated on the basis of the sizes of the polyhedra and virions, at least without rigorous standardization of techniques employed.

TABLE 1
SIZES OF POLYHEDRA AND VIRIONS FROM NUCLEAR-POLYHEDROSIS OF *HELIOTHIS PUNCTIGERA*

Particles Measured	Number Measured	Dimension	Range (nm)	Mean ± S.E. (nm)
Polyhedra	159	Diameter	600-1 750	978 ± 11
Virions with outer membranes	129	Length	210- 700	325 ± 4.9
	129	Breadth	45- 100	76.2 ± 1.1
Virions without outer membrane	21	Length	265- 710	368 ± 18.8
	21	Breadth	38- 64	54.5 ± 1.5
Virions with and without outer membranes	150	Length	210- 710	331 ± 5.1
	150	Breadth	38- 100	73.2 ± 1.1

Difficulty was experienced in sectioning the polyhedra owing to their hardness, at least at their surface, which caused chipping of the glass knife. Sections cut in the correct planes, however, revealed a regular 90° arrangement of the planes of the molecules comprising the crystalline lattice of polyhedral protein. A narrow, dark-staining band was present at or near the periphery in some of the sections of polyhedra, suggesting a double limiting membrane (Figure 1). As the width appeared to be approximately equivalent to that of two layers of the protein lattice, modification of two outer layers of the polyhedral protein lattice may be indicated. Summers and Arnott (1969) observed a similar structure running parallel to the surface of polyhedra within infected cells of *Trichoplusia ni*

TABLE 2
SIZES OF POLYHEDRA AND VIRIONS LIBERATED BY TREATMENT WITH ALKALI OF THE POLYHEDRA FROM *HELIOTHIS PUNCTIGERA*,
HELIOTHIS ARMIGERA AND *HELIOTHIS ZEA*
(Numbers of Measurements are Given in Brackets, where Available)

Virus	Diameters of Polyhedra (nm)		Virions				Alkali Used
	Range	Mean \pm S.E.	Length (nm)		Breadth (nm)		
			Range	Mean \pm S.E.	Range	Mean \pm S.E.	
<i>Heliothis punctigera</i> nuclear-polyhedrosis	600-1 750	978 \pm 11 (159)	210-710	331 \pm 5.1 (150)	38-100	73.2 \pm 1.1 (150)	Na ₂ CO ₃
<i>Heliothis armigera</i> nuclear-polyhedrosis (after Bergold and Ripper 1957)	700-1 200	1 100 mode	—	320 \pm 10†	—	90 \pm 10†	Na ₂ CO ₃
<i>Heliothis zea</i> nuclear-polyhedrosis (after Gregory <i>et al.</i> 1969)	300-2 240	916.4 \pm 9.5 (635)		373 \pm 1.5 (115) 298 \pm 1.3 (484)		75 \pm 0.7 (115) 50 \pm 0.03 (484)	Na ₂ CO ₃ NaOH
(after Tompkins <i>et al.</i> 1969)	600-1 600	1 070 \pm 33 (112)	243-378*	336 \pm 0.9* (599)	47-98*	62 \pm 0.05* (599)	

† Possibly standard deviation.

* Pooled results from treatment with Na₂CO₃ and NaOH.

(Hubn.). These authors suggested that it was possibly related to the polyhedral membrane (Nordin and Maddox 1971), or an artifact resulting from greater deposition of osmium on the surface. Gregory, Ignoffo and Shapiro (1969) suggested that difficulties encountered in dissolving and staining the polyhedra from *H. zea*, as well as their retention of physical shape following extreme physical forces, provided evidence that the outer layer(s) was different from its interior. However, they observed no membrane-like structure or changes in the lattice pattern of the outer layer to support this speculation.

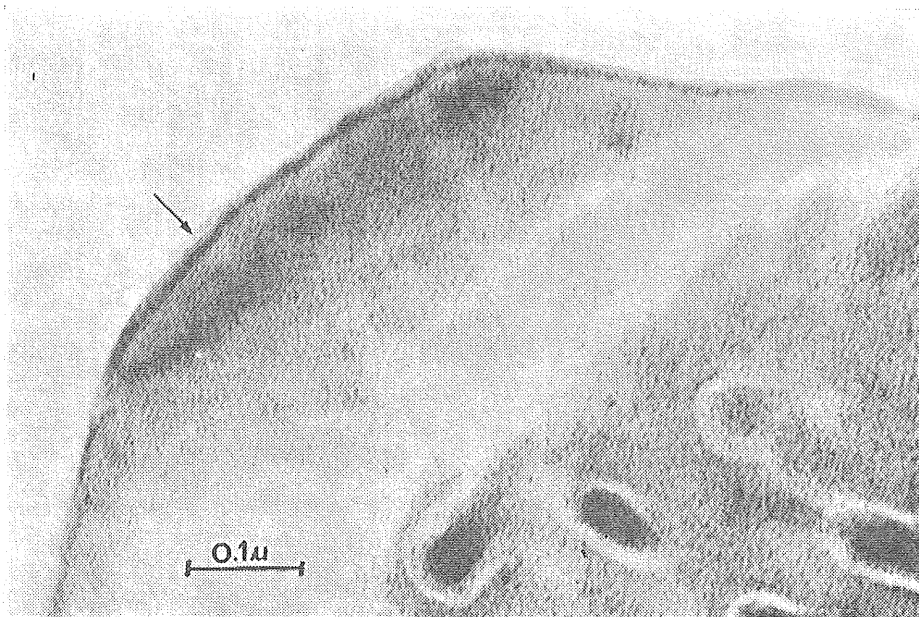


Fig. 1.—Section of polyhedron from *Heliothis punctigera* with dark-staining, limiting, double-membrane-like structure slightly dislodged at the point indicated by the arrow. X190 000.

Virions twice the normal length were occasionally seen in sections of polyhedra and in preparations made by alkali degradation of polyhedra. When the outer membranes were shed, the virions tended to elongate, as is seen in Table 1. This suggests that the inner membrane is a continuous, flexible structure. The surface of the inner membranes had parallel striations or cross-hatching 5.0–5.6 nm apart, similar to the lattice spacing of the polyhedral protein of 5.8 nm. A two or three-tiered nipple structure which appeared to detach readily was frequently seen at one end of virions in alkali-degraded preparations. It was not apparent in the sections of the polyhedra, which suggests that it might have been extruded or revealed by dissolution of surrounding material. This structure may represent a point for the attachment of the virion to the outer membrane or to a susceptible cell, or a zone of weakness through which the infective material is released. A similar structure was seen on virions from *Anthela varia* (Teakle 1969).

(b) Serological Relationships

An attempt was made to determine whether the nuclear-polyhedrosis virus from *H. punctigera* could be distinguished from those from *H. armigera*, *Orgyia anartoides*, *O. australis*, *Anthela varia* and *Pterolocera amplicornis* using antigenic characteristics of the polyhedra. The antigens consisted of solutions of the purified polyhedra in dilute alkali, and antiserum to the *H. punctigera* polyhedral antigen was prepared in guinea pigs.

Agar gel diffusion tests were run with the concentration of the reagents and conditions of the test adjusted to give, in most instances, a single, well-defined precipitin line for each antigen-antibody reaction. There was a tendency for the *H. punctigera* antigen to give quite a strong second line of precipitation. This was closer to the antigen well and may have been due to incomplete breakdown of the protein lattice, or antigenic material represented by the dark-staining band at the periphery of the polyhedron in Figure 1, or the liberated virions.

No reactions were given by antigens prepared from normal host insects, or when non-immune guinea pig serum was used.

Each of the antigens reacted strongly with the *H. punctigera* virus antiserum so that patterns of 'identity' (fusion) or 'partial identity' (partial intersection) (Crowle 1961, p. 70) were obtained (Figures 2 and 3). The results indicated that the antigen preparation from each virus shared a common antigenic component and that the *H. punctigera* and *H. armigera* viruses possessed, in addition, more specific antigenic components which were indistinguishable from each other (Table 3).

TABLE 3

AGAR GEL DIFFUSION REACTIONS GIVEN BY PAIRS OF ANTIGENS PREPARED FROM NUCLEAR-POLYHEDROSIS VIRUSES FROM *HELIOTHIS PUNCTIGERA*, *HELIOTHIS ARMIGERA*, *ORGYIA ANARTOIDES*, *ORGYIA AUSTRALIS*, *ANTHELA VARIA* AND *PTEROLOCERA AMPLICORNIS* WITH ANTISERUM TO *HELIOTHIS PUNCTIGERA* NUCLEAR-POLYHEDRA PREPARED IN GUINEA PIGS

Antiserum	Antigen Pair											
	Hp	Hp	Hp	Hp	Hp	Ha	Ha	Ha	Ha	O	O	Oau
	Ha	O	Oau	A	P	O	Oau	A	P	Oau	A	A
<i>Heliothis punctigera</i> nuclear-polyhedrosis virus	I	Pi (Hp)	Pi (Hp)	Pi (Hp)	Pi (Hp)	Pi (Ha)	Pi (Ha)	Pi (Ha)	*	I	I	I

Pi —reaction of partial identity; virus showing additional specific antigenic group is given in brackets.

I —reaction of identity.

Hp —*Heliothis punctigera* nuclear-polyhedrosis virus.

Ha —*Heliothis armigera* nuclear-polyhedrosis virus.

O —*Orgyia anartoides* nuclear-polyhedrosis virus.

Oau—*Orgyia australis* nuclear-polyhedrosis virus.

A —*Anthela varia* nuclear-polyhedrosis virus.

P —*Pterolocera amplicornis* nuclear-polyhedrosis virus.

* Reaction not given as deterioration of antigen P was apparent at time of testing.

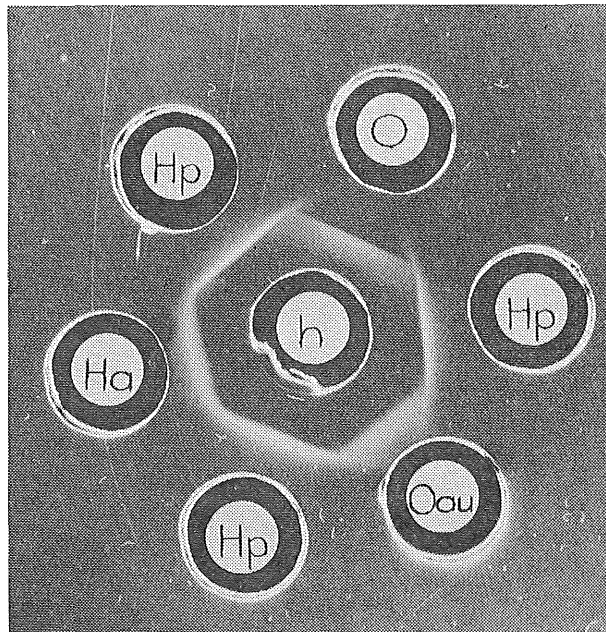
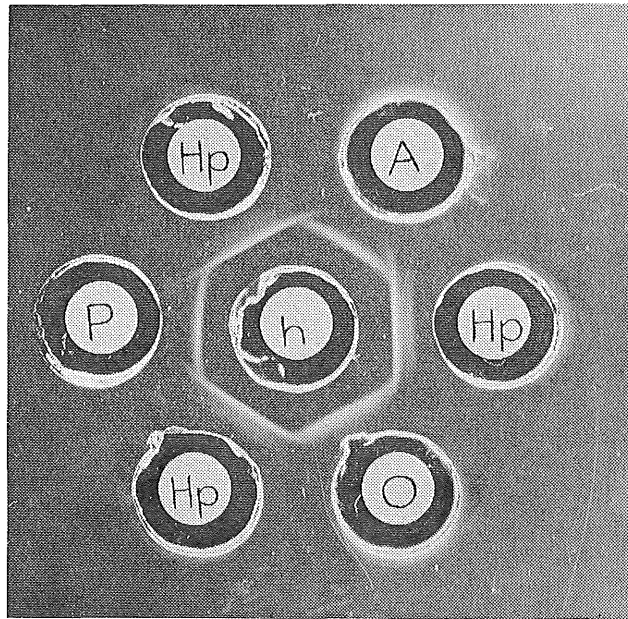


Fig. 2-3.—Agar gel diffusion tests using antigens of nuclear-polyhedra dissolved in dilute alkali in outer wells and antiserum to *Heliothis punctigera* nuclear-polyhedra prepared in guinea pigs (h) in the centre well.

Antigens—Hp—from *Heliothis punctigera*
 Ha—from *Heliothis armigera*
 A—from *Anthela varia*
 O—from *Orgyia anartoides*
 Oau—from *Orgyia australis*
 P—from *Pterolocera amplicornis*

No attempt was made to isolate the antigen, but the serological results, and those obtained with an antiserum to the *O. anartoides* virus (Teakle 1973), were consistent with the existence of a single polyhedral protein for each virus. The proteins from different viruses have both similar and distinct antigenic determinant groups.

Krywienczyk and Bergold (1961) investigated the serological interrelationships of the polyhedral proteins from a number of different host species using the agar gel diffusion technique. They found that polyhedral proteins from Lepidoptera shared at least two antigenic groupings and possessed at least one specific antigenic group characteristic of the individual protein. Their attempt to relate the serological characteristics of the polyhedral proteins to the classification of the hosts was unsuccessful.

(c) Pathology

The symptoms and signs were normal for nuclear-polyhedrosis (Aizawa 1963). The larvae usually died attached to a thin silk mat near the top of the rearing container. The skin was very fragile and ruptured readily (Figure 4). The cadavers were rapidly invaded by bacteria and underwent decomposition.



Fig. 4.—*Heliothis punctigera* larvae killed by nuclear-polyhedrosis. The skin of the larva on the right has ruptured, releasing the body contents containing large numbers of polyhedra.

The infected tissues of fifth instar larvae were first examined by light microscopy, both by phase contrast and as stained sections. The first signs visible after 2 days were small polyhedra in some cells of the midgut (Figure 5), swollen nuclei in some haemocytes and granular areas in the fat body. No indication of infection of the midgut was observed subsequently by light microscopy. At 3 days a low percentage of cells of the fat body contained polyhedra, usually in the 'ring zone', and nuclei of the hypodermis and tracheal matrix were hypertrophied. At 4 days approximately half the cells of the fat body, hypodermis

and tracheal matrix contained polyhedra. The tissues had a spotted appearance due to the presence of cells packed with polyhedra and they disintegrated readily when handled. Some cells in the muscular sheath and the linings of the ventral nerve cord contained polyhedra and free polyhedra occurred in the blood. After 5 days, approximately 75% of the cells of the fat body and hypodermis and 50% of the cells of the tracheal matrix showed polyhedra in stained sections. Infection of the muscular sheath and nerve lining had increased. Death occurred at 5 days.

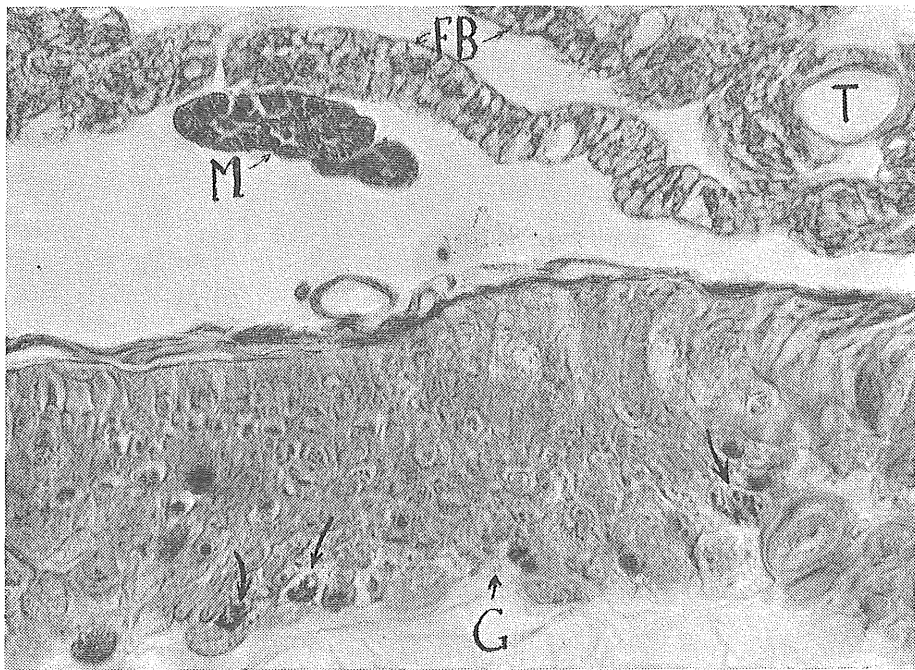


Fig. 5.—Section of *Heliothis punctigera* 2 days after dosing with nuclear-polyhedrosis virus. Arrows indicate cells of midgut containing polyhedra. X600.

The infection of the midgut was confirmed by electron microscopy. Infection of the midgut in this test was not apparent until 3 days after dosing, when nuclei containing virions only, or free virions and polyhedra without embedded virions, were observed. Evidence for the presence of a non-occluded virus was also found in the gut of the larva sampled at 3 days, but not in that of any of the other larvae sampled. The first appearance of infection of the midgut at 3 days coincided with that of the fat body, but no infection of the midgut of the larva sampled at 5 days was observed when heavy infection of other tissues occurred (Figure 6).

Infection of the midgut was also reported for *H. peltigera* (Harpaz and Zlotkin 1965) and *H. zea* (Heimpel and Adams 1966). The apparent absence of infection in this tissue in *H. punctigera* later in the course of the disease

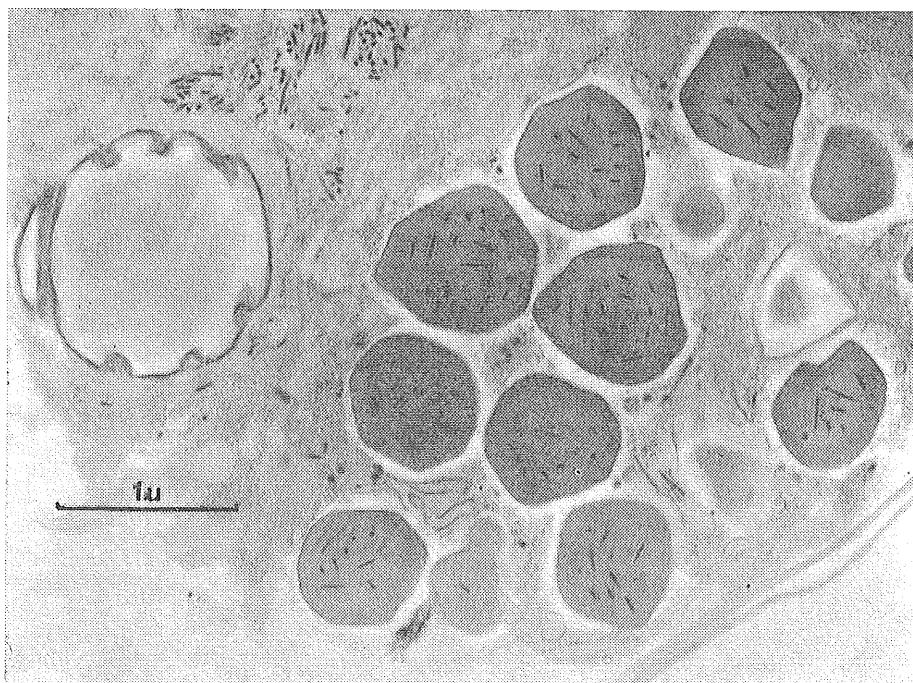


Fig. 6.—Section of tracheole of *Heliothis punctigera* larva 5 days after dosing with nuclear-polyhedrosis virus. Nucleus at upper left contains only virions without the outer membrane. Cell at right contains polyhedra with embedded virions and free virions with the outer membrane. X30 000.

suggested that the infected cells were eliminated, possibly in contaminated faeces. Infected midgut cells discharging their contents or being discarded into the lumen of the gut were observed by Abul-Nasr (1954) in early midgut infections of *Colias philodice eurytheme* Boisd. and *Prodenia praefica* Grote. Abul-Nasr observed infection of the midgut prior to the detection of infection in other susceptible tissues of the two insects and suggested that in a number of instances the later expression of the histopathological signs was prevented by the high regenerative capacity of the intestine. On the other hand, Laudeho and Amargier (1963) described the formation of polyhedra in the nuclei of the midgut epithelium of *Plusia chalcites* Esper. as occurring at the same time as in the other susceptible tissues, and Harpaz and Zlotkin found polyhedra in the midgut of *H. peltigera* only after infection in the other tissues was apparent. The latter authors suggested that the midgut became visibly infected after its regenerative ability had been reduced as a result of the disease. They also observed a number of infected cells with their polyhedron-filled nuclei to be exfoliated into the midgut lumen.

Using the electron microscope Harrap and Robertson (1968) obtained evidence that the midgut of *Aglais urticae* (L.) was a site of early virus production. As observed in *H. punctigera*, the infection was abnormal in that incorporation of the virions into polyhedra was not observed although crystalline protein was present. Subsequently virions were found in the cytoplasm between the nucleus and the basal cell membrane as if moving along a gradient. Presumably

release of the virions into the body cavity resulted in the infection of the other susceptible tissues. Similar findings were reported in *Lambdina fiscellaria fiscellaria* (Guen.) (Cunningham 1971) and *Pseudoplusia includens* (Walk.) (Livingston and Yearian 1972).

In addition to the examination of infected larvae, sections of three 5 to 7-day-old pupae from larvae previously dosed with virus were examined. In two specimens heavy infection of the hypodermis and tracheal matrix occurred, but no infection occurred in the fat body in one of these specimens and there was a markedly reduced level of infection of the fat body in the other. The third pupa showed no sign of infection. Vail and Gough (1970) also noted reduced susceptibility to infection of the pupal fat body of *Trichoplusia ni*, possibly associated with drastic morphological changes in the fat body at metamorphosis.

(d) Infectivity Tests

Tests were carried out on batches of approximately 10 larvae per treatment to determine the influence of larval age (instar) and virus dosage on incubation period and larval mortality.

Effect of larval age (instar) on incubation period.—The incubation periods for each larval instar after comparatively large dosages of 5×10^8 polyhedra per larva are given in Table 4. All larvae of the first five instars tested (1 to 7-day-old larvae) succumbed to polyhedrosis, whereas two sixth (final) instar larvae survived the dosage which is shown in Table 5 to be 5 000 times that which gave virtually complete mortality in fifth instar larvae. Three infected final instar larvae pupated and then succumbed to the infection. Infection in the final instar larvae appeared to prolong larval life. For normal larvae the time to pupation ranged from 5 to 8 days with a mean of 6.6 days based on eight individuals. For infected larvae the range of incubation periods was 10 to 14 days with a mean of 12.3 days based on three individuals. The difference was highly significant ($P < 0.001$).

On the basis of the incubation periods the younger larvae were the most susceptible, whereas the final instar (8 to 9-day-old) larvae exhibited a high degree of tolerance to the virus. Similar relationships between other species of *Heliothis* and their nuclear-polyhedrosis viruses have been demonstrated (Coaker 1958; Ignoffo 1966a, 1966b). Vandamme and Angelini (1966) reported that nuclear-polyhedrosis viruses from *H. zea* and *H. virescens*, which were highly infective for *H. armigera* larvae, produced complete mortality of 8-day-old *H. armigera* larvae but no mortality of 12-day-old larvae. Studies by Allen and Ignoffo (1969) indicated that the apparent increase in resistance to virus infection as larvae mature may be partially explained by the normal increase in body-weight, which may serve to 'dilute' a constant virus dosage. They suggested that resistance which developed before or during prepupation could be due to a change in cell susceptibility as a result of metamorphosis and/or the presence of a viral inhibitor.

From the incubation periods and lengths of larval instars in the present study it was apparent that infection of first instar larvae could result in four cycles of virus production in the one larval generation. The rate of spread in a population of high density could also be enhanced by the cannibalistic tendency of *H. punctigera* larvae and possible early contamination of faeces with virus due to the discharge of infected cells into the lumen of the midgut.

TABLE 4
INCUBATION PERIODS OF NUCLEAR-POLYHEDROSIS OF *HELIOTHIS PUNCTIGERA* DETERMINED FOR EACH INSTAR

Insect Stage at Treatment	Dosage (polyhedra)	Incubation Period (days)		Number of Larvae Dosed	Number Dead		Number Moulded (or Pupated)	Number of Days Infected Before Moulting (or Pupating)
		Range	Mean		Polyhedrosis	Other Causes		
Egg (surface-contaminated)	12.5×10^9	2-4.5	3.7	4	3	1	0	
Larva 1st instar	5.0×10^8	2-4	2.75	10	8	2	8	3
2nd instar	"	4	4.0	10	7	3	10	1
3rd instar	"	4-5	4.2	10	10	0	10	1-2
4th instar	"	4-6	5.3	10	7	3	10	1-2
5th instar	"	4-6	5.1	9	9	0	9	2
6th instar	"	10-14	12.3	10	3*	2	5†	6-8

* 1 larva and 2 prepupae.

† Two subsequently emerged as apparently normal adults at 20 and 21 days, respectively. The remaining 3 pupae failed to emerge and smears of the body contents showed polyhedra.

Effect of virus dosage on incubation period and mortality.—Dosages containing from 10^2 to 10^8 polyhedra were fed to fifth instar (7 to 8-day-old) larvae. The results are given in Table 5.

TABLE 5

EFFECT OF VIRUS DOSAGE ON INCUBATION PERIOD OF NUCLEAR-POLYHEDROSIS AND MORTALITY IN FIFTH INSTAR *HELIOTHIS PUNCTIGERA* LARVAE

Dosage (polyhedra)	Number of Larvae Dosed	Number Dead		Number Pupated	Incubation Period (days)		Mortality (%)
		Polyhedrosis	Other Causes		Range	Mean	
0 (Control)	9	0	0	9	—	—	0
10^2	10	0	1	9	—	—	0
10^3	9	3	0	6*	5-9	7	44
10^4	10	10	0	0	4-14	6.4	100
10^5	6	5	1	0	5-8	6.0	100
10^6	9	9	0	0	4-7	5.3	100
10^7	10	10	0	0	4-14	5.8	100
10^8	8	7	0	1	4-5	4.5	87.5

* One pupa died of polyhedrosis.

A decline in mean incubation period over the dosage range of 10^3 to 10^8 polyhedra was obtained. With one exception, dosages of 10^4 or more polyhedra were lethal for fifth instar larvae under the conditions of laboratory infection.

Chauthani, Claussen and Rehnberg (1968) found a dosage of 7 548 polyhedra from *H. zea* gave a 95% mortality in third instar (6-day-old) *H. zea* larvae. Allen and Ignoffo (1969) reported that dosages of 300, 9 587 and 139 632 polyhedra gave 90% mortality in 6, 7 and 8-day-old *H. zea* larvae respectively.

Cross-infectivity tests.—The virus did not infect larvae of *A. varia*, *O. anartoides*, *Cactoblastis cactorum* (Berg), *Pieris rapae* (L.), *Plusia chalcites* (Esper) and *Spodoptera mauritia* (Boisd.) but was infective for *H. armigera* larvae.

Reciprocal infectivity of *H. punctigera* larvae by a virus from *H. armigera* also occurred. Testing also indicated that *H. punctigera* was not susceptible to a virus from *A. varia* and probably not susceptible to a virus from *O. anartoides*. In one of two tests using the latter virus, no polyhedrosis resulted in the 11 *H. punctigera* larvae dosed, but in the other, 8 out of 9 *H. punctigera* larvae died of polyhedrosis. Alkaline extracts of the dead larvae were made and tested by agar gel diffusion using *O. anartoides* and *H. punctigera* virus antisera to determine the serological identity of the virus present. Extracts from only two of the larvae gave satisfactory precipitin lines, but it was indicated that the virus was not of the *O. anartoides* type but gave identical reactions to those of virus from the larvae dosed with *H. punctigera* virus in the same infectivity test (Figure 7). If the serological identity of the polyhedral protein is a property of the virus and not of the host, the evidence suggests that accidental infection of the *H. punctigera* larvae with *Heliothis* virus or activation of a latent *Heliothis* virus in the larvae had occurred.

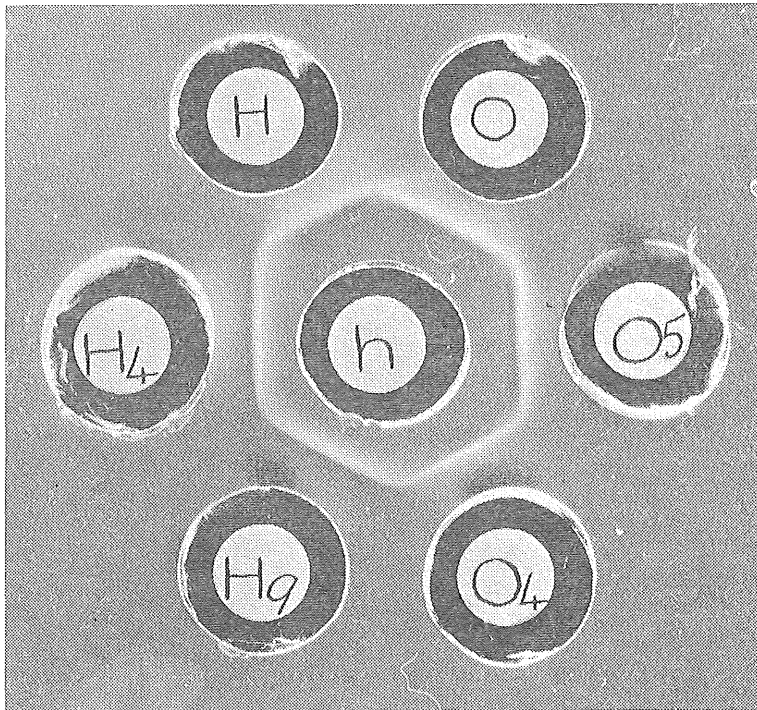


Fig. 7.—Agar gel diffusion test using antigens of nuclear-polyhedra dissolved in dilute alkali in the outer wells and guinea pig antiserum to *Heliothis punctigera* nuclear-polyhedra (h) in the centre well.

Antigens—H from *Heliothis punctigera*

O from *Orgyia anartoides*

H₄ and H₉ from two *H. punctigera* larvae previously dosed with *H. punctigera* nuclear-polyhedrosis virus

O₄ and O₅ from two *H. punctigera* larvae previously dosed with *O. anartoides* nuclear-polyhedrosis virus.

The results suggest that the deaths of larvae O₄ and O₅ were due to *H. punctigera* nuclear-polyhedrosis virus, and not the *O. anartoides* nuclear-polyhedrosis virus.

Quarantine restrictions prevented the import of viruses from *Heliothis* species from outside Australia for testing for infectivity to *H. punctigera*, but extensive cross-infectivity of viruses from *Heliothis* species for other species of *Heliothis* has been reported. Viruses from *H. zea*, *H. virescens*, and *H. peltigera* were found to cross-infect larvae of *H. armigera*, *H. zea*, *H. virescens*, *H. paradoxa* (Grote) and *H. ploxiphaga*. Nine species in other genera were not susceptible (Ignoffo 1968). Tompkins, Adams and Heimpel (1969), however, found that one of two types of nuclear-polyhedrosis virus obtained from *Trichoplusia ni* could be transmitted to *H. zea*, but the infection differed from that of the normal *H. zea* nuclear-polyhedrosis in that the cells of the midgut epithelium were infected but those of the fat body were rarely infected.

The reciprocal cross infectivity of the viruses from *H. punctigera* and *H. armigera* and the antigenic similarity of their polyhedral proteins suggest that they may be, in fact, the same virus. As mixed populations of these insects occur in coastal areas of Queensland (Kirkpatrick 1961), exchange of virus between these species undoubtedly occurs.

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