THREE OCCLUDED INSECT VIRUSES

A biophysical and biological study
of the nuclear-polyhedrosis virus of *Colias electo*
the granulosis virus of *Heliothis armigera*
and the nuclear-polyhedrosis virus of *Heliothis zea*

by

HELA GITAY (neé DIAMANT)

A thesis presented in the fulfilment
of the requirements for the degree of

MASTER OF SCIENCE

of

UNIVERSITY OF CAPE TOWN

June, 1971

This work was carried out in the M.R.C. Virus Research Unit in the Department of Bacteriology, Medical School, University of Cape Town.

All the experimental work, including the electron microscopy and the photography, was carried out by the author.

CONTENTS

CHAPTER PAGE

CHAPTER PAGE

SUMMARY

An investigation was undertaken in some detail of three virus strains of insect pests of agricultural importance, viz. a nuclear-polyhedrosis virus of the lucerne caterpillar, Colias electo, and a granulosis virus of the bollworm, Heliothis armigera, both found in South Africa, and a nuclear-polyhedrosis virus of the bollworm, Heliothis zea, isolated in America, with a view to ascertaining a knowledge of some of the fundamental properties and basic biology of these infective agents. On the basis of the information gained the viruses could be differentiated and their broad classification was established.

The morphology of the polyhedra, capsules and virus particles observed by light and electron microscopy has been completed and measurements of the viral elements have been made.

Some biophysical properties of the virus particles and their inclusion bodies were recorded, i.e. their resistance to chemical and physical treatments and their relative mobility in an electric field under standard conditions.

Observations were made on procedures which brought about varying degrees of purification and concentration of the virus particles from putrefying larvae and the most successful of these were found to be reproducible. They involved the purification of the inclusion bodies and their digestion by weak alkali to release the virus particles. Both preparations of the viral elements were further

purified by zone electrophoresis in sucrose density gradients.

Some information was gathered on the mode of transmission of the infection from insect to insect by contact or cannibalism, from one generation to the next through the eggs, and particularly from one area to another by virus survival in avian faeces.

The incidence and rate of the infection in the larvae was increased by environmental changes such as raising the temperature and also to some extent by spraying with a suspension of endospores of Bacillus thuringiensis. Exposure to other stress conditions was not successful in initiating a fatal infection in the insects. Of particular interest, however, was the observation that by injecting a 'foreign' virus a fatal infection was induced by activation of a native occult virus in the larvae of the silkworm, Bombyx mori.

In the context of the possible application of these infective agents to future methods of biological control of economically disastrous pests, these preliminary experiments were not unrewarding.

CHAPTER 1

INTRODUCTION

Insect viruses are classified as 'occluded' or 'nonoccluded'. The occluded viruses, some of which are dealt with in this study, possess inclusion bodies and constitute 95 percent of the known insect viruses. They are subdivided into polyhedrosis and granulosis viruses. The agents responsible for polyhydrosis virus infections occur in the form of aggregates of virus particles lying singly or in bundles in a regular matrix of proteinaceous material termed a polyhedron. The granulosis infective agent has a smaller ovoid inclusion body known as capsule or granule containing only one virion or rarely two. The polyhedral viruses are further subdivided into nuclear and cytoplasmic forms according to the site of replication; they also differ in being either rod-shaped or spherical in form.

Occluded insect viruses were among the first viruses to be investigated because the inclusion bodies were large enough to be seen under the ordinary light microscope. The silkworm, because of its great economic importance, was the first insect in which a study of an insect virus disease was made. Thus, in 1856, Cornalia and Maestri observed polyhedra in the silkworm and concluded that these highly refractile bodies of crystalline outline represented

an infective agent. It was not until 1894 that Bolle established the proteinaceous nature of the inclusion bodies and suggested that they were the carriers of the infective agent rather than the agent itself. In 1907, Von Prowazek showed that the infective agent was a filterable virus by demonstrating the infectivity of a filtrate from which inclusion bodies had been removed, but he did not realize the relationship of the filterable virus to the inclusion bodies. Later, the development of dark field microscopy enabled Komárek and Breindl in 1924 to observe the Brownian motion of bundles of virus particles emerging from the polyhedra after the dissolution of the latter in alkali. Final confirmation came in 1947 when Bergold managed to isolate the virus particles from the polyhedra of the silkworm and other insects and to observe the detail of their structure under the electron microscope (Bergold, 1953). The polyhedrosis viruses of many other species of Lepidoptera, Hymenoptera and Diptera were subsequently found to be broadly similar in structure to those of the silkworm.

Viruses belonging to the granulosis group, however, have only small inclusion bodies which are barely visible by light microscopy. They were discovered later than the nuclear and cytoplasmic-polyhedra and it was only in 1926, by using dark field microscopy, that Paillot defined and described the capsular structure of the granulosis inclusion bodies of the large white butterfly and concluded that these capsules were the infective agent. Very few of these granulosis virus infections were investigated until the

electron microscope became available and it was only then that the capsules were recognized as the carriers of the infective virus rather than the virus itself. Steinhaus (1947) was the first to record the presence of the granulosis virus in America. Bergold, in 1948 (Smith, 1967) presented the first electron micrograph of capsules. Steinhaus *et al.* (1949) described the rod-shaped virus within the inclusion body and concluded, by analogy with nuclear-polyhedral viruses, that this was the infective agent. Granulosis viruses were later found in other insects, but only in the order Lepidoptera and predominantly in the families Pieridae and Noctuidae.

A binomial classification of insect viruses has not yet been developed. The first attempt at classification based on electron microscopy was made by Steinhaus (1949) and it was based on the shape and the presence or absence of inclusion bodies. He proposed four genera: *Borrelina* (Paillot) for polyhedral inclusions, *Paillotela* (Steinhaus) for refringent polymorphic inclusions, *Bergoldia* (Steinhaus) for granules and *Morator* (Holmes) for viruses without inclusion bodies.

Bergold (1953) introduced the criterion based on the shape of the virus particles, describing the *Borrelina* and the *Bergoldia* genera as rod-shaped infective units, the *Morator* genus as spherical ones and additionally included the genus *Smithia* (Bergold) consisting of spherical viruses occluded in polyhedra.

Later, using the criteria of the primary site of virus

replication in the cytoplasm or nucleus of the cell and the tissues infected, nine genera of insect viruses were established, as summarized by Ignoffo (1968). Incomplete lists of classification are given for nuclear-polyhedrosis viruses (Bergold, 1963a) for cytoplasmic-polyhedrosis viruses (Smith, 1963) and for granulosis viruses (Huger, 1963). Classification of the nonoccluded viruses is even more incomplete.

Modification of the proposed classification is inevitable because new virus diseases of insects are continually being discovered and viruses which do not fit well into any of the taxonomic categories have also been described (Smith and Xeros, 1954a; Vago, 1963). In some instances, viruses have been named and classified according to the insect host but in the absence of host specificity, this has been misleading.

The present study was stimulated by an interest in the basic structure of these viruses and in the possibility of using insect viruses in the biological control of some common South African insect pests, in particular the bollworm and the lucerne caterpillar.

Three different viruses were investigated; the nuclear-polyhedrosis virus of the South African lucerne caterpillar, Colias electo, the nuclear-polyhedrosis virus of the bollworm, Heliothis zea, isolated in America, and the granulosis virus of the bollworm, Heliothis armigera, found in South Africa.

The wilt disease of the lucerne caterpillar, Colias

electo, was first described by Mally (1908) and Lounsbury (1913). They found that control of these caterpillars was achieved by spraying an aqueous solution of dead larvae on the lucerne plants. This was the first instance of biological control using the agent of a naturally occurring infection in insects, even although the infective agent was not recognized until many years later. In 1936 Smith had suggested that this wilt disease was of bacterial origin but Tripconey (1969) isolated the virus of Colias electo wilt disease and identified it as a nuclear-polyhedrosis virus. In America the related species, the alfalfa caterpillar, Colias eurytheme, (Steinhaus and Thompson, 1949) has been successfully controlled by the use of its nuclear-polyhedrosis virus.

The wilt disease of the bollworm, Heliothis zea, was described by Chapman and Glaser (1915) and by Stahler (1939); the causal nuclear-polyhedrosis virus was first demonstrated by electron microscopy by Smith and Rivers (1956). Further electron microscopy studies of this virus were carried out by Bergold and Ripper (1957) and Gregory et al. (1969). The effectiveness of this virus in biological control was described by Ignoffo et al. (1965).

With regard to the bollworm, Heliothis armigera, a description of granulosis virus infection in this insect has not been found in the literature. However, symptoms of a wilt disease of the bollworm in South Africa were described by Mally in 1891 (Ignoffo, 1965a) but this could have been due to either nuclear-polyhedrosis or granulosis viruses. In 1936 Parson described the nuclear-polyhedrosis virus

infection of Heliothis armigera (Ignoffo, 1965a) and later a granulosis virus was isolated from Heliothis zea by Falcon et al. (1967). Successful biological control with granulosis viruses has so far been described in Hawaii, New Zealand and France but only of the cabbage pests of the genus Pieris (Huger, 1963; David and Gardiner, 1966; Smith, 1967).

The main object of this study was to investigate the biophysical and biological properties of the local strains of virus because it was felt that a thorough understanding of their nature and behaviour would lead most rapidly and most effectively to the control of their hosts by future biological methods.

CHAPTER 2

THE LIFE CYCLE AND SYMPTOMATOLOGY OF THE HOSTS AND TRANSMISSION OF THE VIRUSES

2.1. INTRODUCTION

2.1.1. The life cycle of Heliothis armigera Hübner (Lepidoptera: Noctuidae).

According to Nel (1958), Heliothis armigera is the most common species of the Heliothis occurring in South Africa. It is very wide spread and occurs in other parts of Africa and in Australia, Europe, India, New Zealand and South Pacific Islands. The common names applied to Heliothis armigera are related to the many crops on which the larval stages feed, e.g. cotton bollworm, tomato fruitworm, lupin worm, corn earworm, peaworm, citrus bollworm, carnation budworm and tobacco budworm. The larvae feed mainly on the fruit and to a lesser extent on the stem and leaves. In lucerne, however, while the newly hatched larvae feed on the internal parts of the flower, the leaves generally are devoured by larvae of the third and subsequent instars. However, some larvae appear to feed on the leaves throughout all their larval stages.

The eggs are laid singly on the plants. They are 0.48 x 0.50 mm in size, shiny, yellowish-white, are dome-shaped and of rough sculpture. The fertile eggs darken towards the time of hatching and the incubation period of

the larvae is 3-5 days in summer and up to 9 days in winter.

At the time of hatching, the larvae are 1.5 mm long and white in colour. They undergo five larval instars and when mature, their average length is 40 mm. The ventral side is usually pale yellowish grey. The colour and the markings occur on the dorsal and lateral sides only. Their colour varies from green, brown and black to that of orange and pink and they may be striped or spotted. Although they usually exhibit black tubercles, some smooth forms occur. The younger larvae are darker and more hairy than the older ones. The larvae are cannibalistic and attack other species, such as the lucerne caterpillar, that occurs together with them in the lucerne fields. The larval period is 14-33 days, depending on temperature and other climatic conditions.

The larvae pupate in the soil at a depth of 1-4 inches in a pupal case which is formed from silk and earth. The pupa, 17-22 mm long, changes colour from yellowish brown to brown and remains in the soil for 13-18 days.

The adult moths fly by night and are rarely seen. The male and the female are differently coloured. The head and thorax of the male are olive-grey with transverse orange-brown lines, whereas the female has a reddish-brown head and a thorax with black transverse lines. The male is usually smaller than the female and lives for an average of 9 days compared to 17 days for the female. The preoviposition stage lasts 2-5 days and the average number of eggs laid per female is approximately 1000. The adults have a tendency to move with the wind in their search for new supplies of

food, resulting in new areas of infestation.

The total life cycle is completed in 32-77 days and varies according to environmental conditions. There are usually four generations per year, in spring, early summer, midsummer and late summer and a fifth generation may result if the autumn is mild. The pupae of this generation in most cases remain in the soil until the following spring, giving rise to a long life cycle of up to 265 days. An abnormally wet winter, as may occur in the Western Province, may cause death of most of the pupae in the soil.

2.1.2. The life cycle of Heliothis zea Boddie (Lepidoptera: Noctuidae).

Heliothis zea occurs widely over North and South America, (Wilcox et al., 1956). It has been confused morphologically with Heliothis armigera and as it also destroys a similar variety of crops it has been given the same common names as Heliothis armigera. According to Common (1953) and Todd (1955), the two insects differ morphologically in wing markings, the number of spines on the foretibia and in the anatomy of the genitalia, the differences being more pronounced in the male. The stages of the life cycle of Heliothis zea are similar to those of Heliothis armigera but, due to differences in the local environment, they tend to be rather longer in nature. When reared in the laboratory on a semisynthetic diet at a temperature of 24 - 30°C (Ignoffo, 1965b), Heliothis zea has a shorter life cycle than either species in nature. Comparative details of the duration of life cycle and the

relevant stages are given in Table 2.1.

The life cycle of Heliothis zea in nature, being 46-110 days, permits only two or three generations per season. However, because of the favourable environment throughout the year and the consequent shorter life cycle, more generations per year are possible in laboratory reared insects.

2.1.3. The life cycle of Colias electo Linnaeus (Lepidoptera: Pieridae).

Species of the genus Colias are common pests in America, Europe, Asia and Africa. The nominotypical form, Colias electo electo, occurs only in South Africa where it is very common and is reputed to be the main pest of lucerne (Smit, 1936). Other races are abundant in Africa, Europe, Asia and the Pacific Islands (Van Son, 1949). In South Africa the adult Colias electo is known as the African clouded yellow butterfly (Clark and Dickson, 1952). The Colias electo larva is known as the lucerne or alfalfa caterpillar. It is the larval stage that is destructive as it feeds mainly on leaves of lucerne and clover plants.

The eggs, 1.1 mm long and 0.4 mm wide, have a pale salmon colour with a yellow end. They are laid singly on leaves, buds or stems of lucerne plants and hatch after 4-10 days.

The emerging larva is 1.5 mm long, pale olive with a black head. During five instars it grows up to 30 mm and changes colour to green with a white stripe along the lateral sides, although some larvae have additional dark

green and broken black stripes. The skin is covered by spines. Unlike the Heliothis species, the larvae taper towards the tail. The larval period is 20-25 days in spring, summer and autumn and 38-70 days in winter.

Pupation takes place on the leaves and the pupal stage lasts for 7-11 days in summer and 17 days in winter. The pupae are 15-20 mm long and green in colour.

The ventral surface of the butterfly is yellowish green and the dorsal side of the wings is spotted orange. The female is darker than the male. The longevity of the female butterfly is about 7 days and the preoviposition 1-2 days. The female lays up to 75 eggs on the young lucerne leaves.

The total life cycle is 32-104 days with five to seven generations a year. Clark and Dickson (1952) and Van Son (1949) report that this species occurs in South Africa all through the year. In the vicinity of Cape Town, however, the larvae were found mainly during spring and summer.

Table 2.1. Life cycle of the investigated insects. Duration in days.

	Heliothis armigera	Colias electo	Heliothis zea	
	In nature	In nature	In nature	In laboratory*
Egg period	3 - 9	4 - 10	5 - 10	3 - 4
Larval period	14 - 33	20 - 70	21 - 44	10 - 11
Pupal period	13 - 18	7 - 17	18 - 33	10 - 12
Oviposition	2 - 17	1 - 7	2 - 23	2 - 21
Total life cycle	32 - 77	32 - 104	46 - 110	25 - 48

* According to Ignoffo (1965b) whose laboratory-reared insects were the source of the virus used in the present study.

Observations made while collecting specimens indicated that the cattle egret, Arbeola ibis, was a predator of the insect pests. Accordingly, it was decided to collect specimens of avian faeces from these predators and attempt to recover virus from this source.

The recovery of virulent nuclear-polyhedra from the faeces of other birds has been reported by Vago and Bergoin (1968) and by Hostetter and Beiver (1970). The latter authors point out that the sick larvae fall an easy prey to the birds as their colour is more distinguishable and they tend to move to exposed parts of the plant. Smit (1936) reports the destruction of large numbers of Colias electo caterpillars by the large European stork and by the locust bird. The examination of the faeces of these birds for viral agents should be rewarding but was not possible during this investigation.

Histopathological studies were not carried out on the infected insects but it is generally believed (see below) that the pathogenesis of the lesions occurs in the following ways. The insect is usually infected by ingestion and the inclusion bodies are dissolved by the gut juice, releasing the virus particles. The particles pass into the haemocoel through the epithelial cells of the midgut and travel to the susceptible cells. In the case of nuclear-polyhedrosis virus infection, replication takes place in the nucleus. The nucleus expands, the chromatin forms a net which is termed the virogenic stroma (Xeros, 1956) and in the stroma virus formation starts. The resultant virions then travel

to the periphery of the nucleus, known as the ring-zone and there they are enclosed within their membranes and polyhedra. The biosynthesis of the polyhedral protein occurs in the cytoplasm (Adams and Wilcox, 1968). When about 100 polyhedra are formed the cell bursts, releasing its contents into the haemocoel (Bergold, 1953). Free, nonoccluded virus particles can then attach new cells.

There had been some doubt in the past regarding the site of the granulosis virus replication, because originally it was observed in the nucleus (Smith and Xeros, 1954b), in the cytoplasm (Bird, 1959) or in both (Smith and Rivers, 1956). It was established subsequently that the formation of the virus particles takes place primarily in the nucleus which then expands and bursts, with replication continuing in the cytoplasm in material of nuclear origin (Huger and Krieg, 1961; Bird, 1963).

2.2. MATERIALS AND METHODS

2.2.1. Harvesting and rearing Heliothis armigera.

The larvae of the bollworm were collected in infested lucerne fields at Elsenburg, near Cape Town, during December, 1969 - January, 1970. The ambient temperature varied between 18.7°C and 37.2°C. The lucerne plants were vigorously shaken by hand and the caterpillars were allowed to fall into a container. A superficial search in the soil at that time did not reveal any pupae. The bollworm caterpillars were brought to the laboratory and were separated from the other insects in the sample collected.

The Heliothis armigera larvae were reared on lucerne leaves at room temperature in sterilized glass jars, 30 cm diameter and 12 cm high. All failed to pupate and died in the larval stage. The caterpillars were inspected, signs of disease were recorded and drops of haemolymph from sick and healthy insects were checked for the presence of viral inclusion bodies under the light and the electron microscopes.

2.2.2. Harvesting and rearing Colias electo.

Caterpillars and pupae of Colias electo were found earlier in the season, starting in November, 1969 and were collected in the same lucerne fields as Heliothis armigera. The ambient temperature in November varied between 17.3°C and 33.7°C and in December between 18.7°C and 36.0°C. In January, 1970, when the temperature range was 21.3°C - 37.2°C, the lucerne caterpillars became less abundant while the bollworm was the dominant pest in the fields. In the laboratory the same rearing techniques as for Heliothis armigera resulted in pupation of many of the larvae and even a few butterflies hatched out. To obtain a better yield of virus, larvae were reared at 37°C (3.2.1. and 3.2.2.) which gave higher mortality amongst the larvae and caused a higher percentage to die within a few days.

2.2.3. Isolation of virus from avian faeces.

The cattle egret, Arbeola ibis, was seen to feed on the caterpillars in the same lucerne fields at the time the collections were being made. Since most of the larvae were

infected with either nuclear-polyhedrosis virus or with granulosis virus, an attempt was made to isolate these viruses from the avian faeces. Samples of faeces were suspended in water and purified according to the procedure described in 4.2.5. A sample of the final pellet was checked in the electron microscope. The inclusion bodies, being granulosis capsules, were digested with alkali (4.2.7.) and the isolated virus particles were also observed by electron microscopy. They could thus be compared with the granulosis capsules and virus particles isolated from Heliothis armigera.

2.2.4. Source of the nuclear-polyhedrosis virus of Heliothis zea.

The preparation used in this study was a commercially prepared powder marketed as Viron/H by International Minerals and Chemical Corporation, U.S.A., containing 4×10^9 nuclear-polyhedrosis inclusion bodies per gram. Other ingredients present in the preparation were an inert powder diluent, residues from dead larvae of Heliothis zea and bacterial contaminants.

2.3. RESULTS

2.3.1. Manifestation of disease in the infected insects.

Colias electo and Heliothis armigera larvae died within 1-10 days after they were brought to the laboratory. They showed overt signs of disease only 2-3 days before death. Cessation of feeding, sluggishness, flaccidity and changes in colour, in this order of appearance, were noticed in the two pests. The aggressiveness and cannibalism of Heliothis armigera larvae decreased as they became more sluggish. The colour varied with the species. The lucerne caterpillars changed from green to yellow and then to dark brown when they discharged drops of brown fluid. In the bollworms, first the ventral part became yellowish white and this later spread all over the body. The fluid discharge from the bollworms was milky white and opaque. In both species of caterpillar the fluid discharge as it dried, caused the insects to adhere to the jar or to the plants. Occasionally, they adhered to the plants head down, either linearly held by their prolegs, or in an inverted V shape held by the abdominal legs. The fragile skin ruptured easily, releasing the liquefied body contents, brown from Colias electo and milky white from Heliothis armigera. If left in the jar too long, both species of caterpillar eventually dried up completely, but inclusion bodies could still be recovered from them.

The sick Colias electo pupae were dark brown compared with the green of the apparently healthy insects. Their body contents, though liquefied, could not leak out from the

Fig. 1. *Colias electo* larvae. Centre - healthy green larvae. Left and right - diseased larvae. Note change of colour from green to brown.

Fig. 2. *Colias electo* pupae. Left - healthy green pupa. Right - diseased brown pupa.

Fig. 3. *Colias electo* adults. Left - healthy butterfly. Lower right - infected butterfly with undersized body. Upper right - infected butterfly with undersized body and abnormal wings.


1


2


3

Fig. 4. Heliothis armigera, infected larvae.

Fig. 5. Heliothis armigera, healthy pupa on the right and healthy moth on the left.

thick cuticle and the pupae dried up leaving almost empty shells, which were found to contain polyhedra. No bollworm reached the pupal stage.

In the majority of instances, normal looking pupae gave rise to healthy butterflies and of the examined insects only once could polyhedra be demonstrated. Some adults failed to emerge from normal looking pupae even although no polyhedra were found. The obviously sick adults were usually undersized, often with abnormal or underdeveloped wings and failed to mate or lay eggs.

Figures 1-3 show the difference in appearance between sick and healthy insects in the larval, pupal and adult stages of Colias electo. Figure 4 shows the difference in appearance of sick Heliothis armigera according to the variations in their natural colour pattern. Healthy pupa and moth of Heliothis armigera obtained from a laboratory reared stock (kindly supplied by Dr. S.S. Walters, Dept. Entomology, University of Stellenbosch) are presented in Fig. 5.

2.3.2. The virus isolated from the avian faeces.

Intact granulosis capsules were isolated from the cattle egret faeces, as seen in Fig. 6. Comparison with the capsules isolated from the bollworm (Fig. 7) shows a similar picture. The free virus particles, as shown in Fig. 8, are identical with the ones isolated from the larvae (Fig. 9). No nuclear-polyhedra were observed (Gitay and Polson, 1971).

Figs. 6-7. Electron micrographs of negatively stained granulosis capsules. Magnification X 100,000.

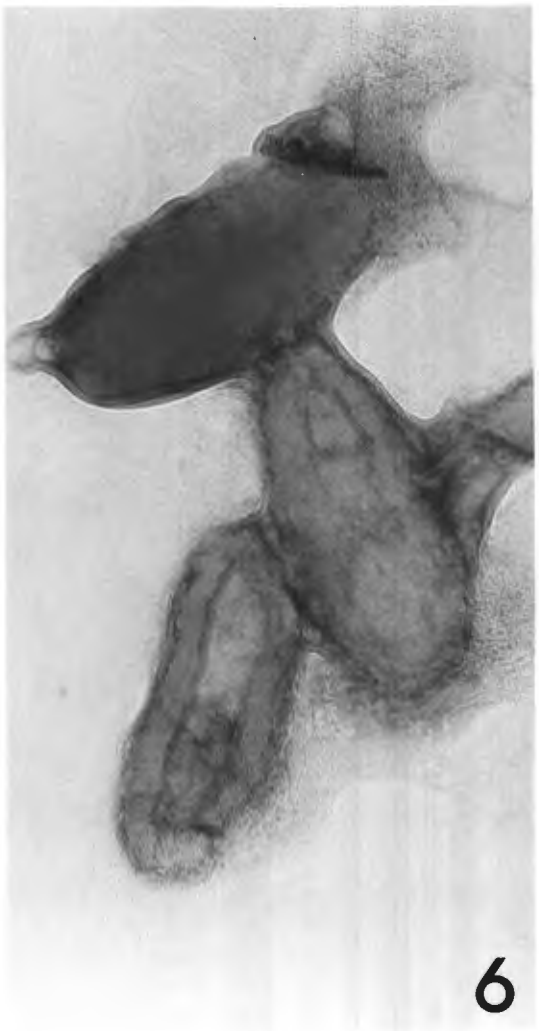
Fig. 6. Capsules isolated from cattle egret faeces.

Fig. 7. Capsules isolated from Heliothis armigera larvae.

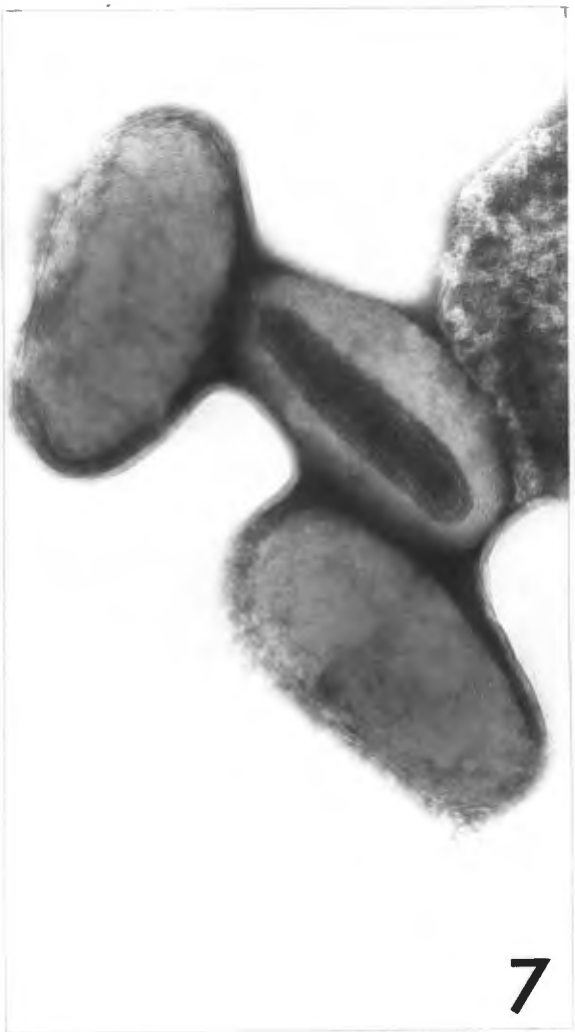
Figs. 8-9. Electron micrographs of negatively stained granulosis virus particles within their outer membrane. Magnification X 100,000.

Fig. 8. Virus particles obtained from digested capsules isolated from cattle egret faeces.

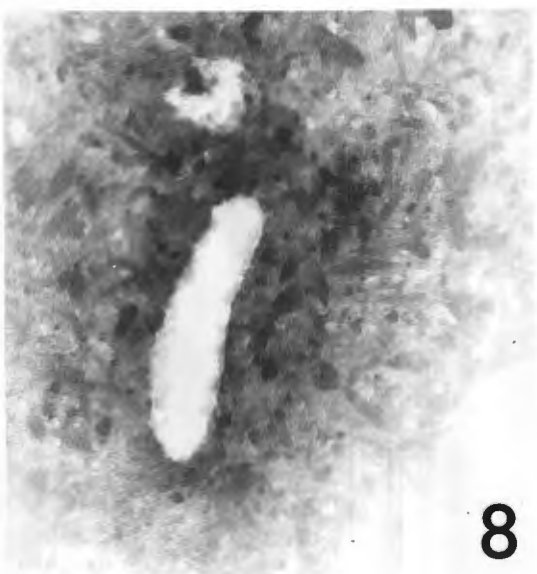
Fig. 9. Virus particles obtained from digested capsules isolated from Heliothis armigera larvae.



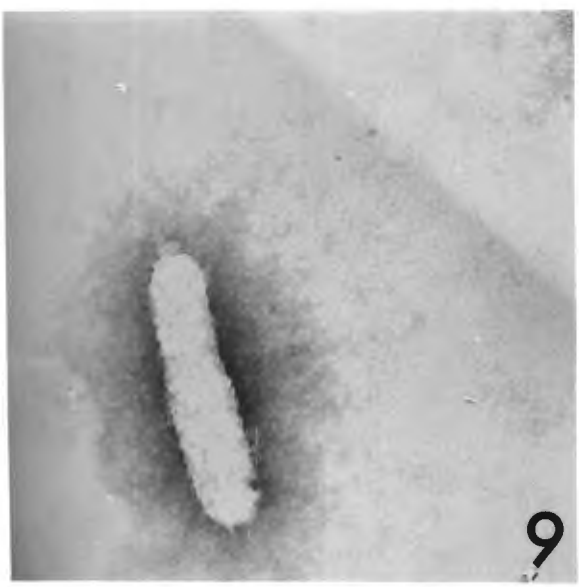
6



7



8



9

2.4. DISCUSSION

Larvae of the bollworm, Heliothis armigera, and of the lucerne caterpillar, Colias electo, were collected from the same lucerne lands on a farm near Cape Town.

Reared on lucerne leaves in the laboratory, all the bollworm larvae died without pupation. Many of the lucerne caterpillars pupated and a few adult butterflies hatched out. Before death, the behaviour of the larvae was seen to be similar showing cessation of feeding and increasing sluggishness but the colour changes and the discharges were quite different in the two species. Polyhedra could be demonstrated in the sick and seen in some healthy looking larvae, pupae and adults of Colias electo. Granulosis virus was demonstrated in the larvae of Heliothis armigera and in the faeces of the cattle egret, Arbeola ibis, which is a natural predator for the bollworm. It would appear that the granulosis virus of the bollworm is very virulent and may thus be an important factor in the natural control of the insect.

The same symptoms observed in Colias electo larvae infected with nuclear-polyhedrosis virus were described by Lounsbury (1913) and by Tripconey (1969) and are similar to those described in the larvae of the related American species, Colias eurytheme, (Steinhaus, 1948). All nuclear-polyhedrosis virus infections in larvae of Lepidoptera or Hymenoptera are known to be manifest in more or less the same way (Smith, 1967).

Steinhaus (1949) showed that the larvae of Colias eurytheme succumb to the disease more readily than the pupae and butterflies. This susceptibility applies to other pupae (Aizawa, 1963; Vail and Gough, 1970) and to adults (Martignoni, 1964; Vail and Hall, 1969). Bergold (1953) maintains that some pupae and adults are completely unsusceptible.

There is a possibility that the pupae and adults of Colias electo were themselves infected by contact with sick larvae but they may merely carry the virus from the larval stage in sublethal doses. The fact that some normal looking pupae, which did not emerge as adults, did not show the presence of inclusion bodies under the microscope may be explained by a sublethal low virus concentration. That sublethal doses of nuclear-polyhedrosis virus have an effect on adult emergence as well as on moulting and pupation has been observed also by Steinhaus (1949), Morris (1962) and Doane (1967). The effect of sublethal doses of virus on the adults in this study was manifest in a smaller body size, in abnormal wings and absence of fertility. Similar observations were made in the silkworm cytoplasmic-polyhedrosis virus infection (3.3.3.), which are discussed in 3.3.4. Some factors other than dose play a part in the infection since some adults with a normal appearance were found to contain polyhedra, a finding also observed by Doane (1967). On the other hand there is a possibility that the failure of normal looking pupae to emerge as adults may be due to death from other causes including factors in the laboratory environment.

The disease of Heliothis armigera due to granulosis virus has not been reported before this study. Granulosis virus infection of Heliothis zea however, has been reported, but the symptoms differ from those in Heliothis armigera in that the infection did not involve the skin sufficiently to produce leakage of the body contents (Falcon et al., 1967). Other species of Lepidoptera, especially the Noctuidae family, exhibit symptoms of granulosis virus infection similar to those observed in this study (Smith and Rivers, 1956; Huger, 1963; Smith, 1967).

Pupae generally are very rarely infected by granulosis virus and no adults are known to succumb to the disease. The phenomenon of failure of the infected Heliothis armigera larvae to pupate was also observed in granulosis virus infected cutworms (Steinhaus, 1947). Huger (1963) also mentioned that death usually occurs in the larval stage.

The symptoms of the nuclear-polyhedrosis virus infection of Heliothis zea, which were not studied in this work, were observed in larvae of Heliothis zea known by the old names Heliothis obsoleta (Stahler, 1939) and Heliothis armigera (Steinhaus, 1949) and were reported to be similar to those of Colias electo plus the additional signs of a metallic lustre and paralysis.

The difference noted in the appearance of the bollworms and the lucerne caterpillars and their body fluids is due to the effect of the viruses on different tissues. In infections with granulosis viruses the main and primary site

of virus replication is the fat-body and this is responsible for the milky white appearance observed. Cases where only the fat-body is attacked are known (Hamm and Paschke, 1963) but often the epidermis is also a susceptible tissue and occasionally the tracheal matrix is affected (Huger, 1963; Benz, 1963). Granulosis viruses may occasionally infect Malpighian tubules (Tanada and Leutenneger, 1968) or midgut cells (Bergold, 1953).

Nuclear-polyhedrosis viruses have a wider range of susceptible tissues. The fat-body, epidermis, blood cells and tracheal matrix, in this order of frequency, are common targets. Other tissues that might be infected later are muscles, muscular sheaths, nerve sheaths, ganglia and pericardial cells. Cases of nuclear-polyhedrosis virus infection of epithelial cells of the midgut are also known (Smith, 1967) as well as Malpighian tubules, silk glands, gonads and connective tissues of the midgut (Mathad et al., 1968). The presence of the masses of polyhedral inclusion bodies in many of the tissues gives rise to the opaque appearance of the infected insects.

To summarise, the external symptoms of the virus infections are nonspecific. They are very similar for most infections caused by nuclear-polyhedrosis viruses and most granulosis viruses and sometimes for both groups of infections. They are, however, typical enough to signify either in the field or in the laboratory that nuclear-polyhedrosis or granulosis infections are involved. In the case of the viruses in this particular study, the

symptomatology was distinctive.

The transmission of the virus was noticed to take place in several ways. When the liquefied body contents, containing millions of inclusion bodies, were liberated, they spread over the leaves of the crop concerned. Bird (1961) reported that healthy larvae readily eat the leaves contaminated with discharges of the sick larvae. This is typical for infections caused by nuclear-polyhedrosis and granulosis viruses only and thus presents a great advantage in biological control (Smith, 1967). Cannibalism, as found in the bollworm, also helps the transmission of viruses.

The potential role of insect-feeding birds in spreading the granulosis virus was demonstrated by the presence of intact granulosis capsules in the faeces of the cattle egret, Arbeola ibis. The nuclear-polyhedra isolated from Colias electo larvae, collected from the fields in which the birds were feeding, could not be recovered from the avian faeces despite the fact that the eating habits of the cattle egret are non-selective. This was evidenced by counts made of the gastric contents which showed the larvae of Colias electo and Heliothis armigera to be similar in number (Dr. W.R. Siegfried, Institute of Ornithology, University of Cape Town, personal communication). This finding shows the greater stability of the granulosis capsules, as compared to the polyhedra, in the alimentary tract of the bird. Infectivity of the capsules was not tested, but being intact they were presumed to be still infective.

CHAPTER 3

THE EFFECT OF STRESS ON VIRUS INFECTION

3.1. INTRODUCTION

Farmers in the Stellenbosch and Oudtshoorn districts have reported to us that caterpillars sicken and fall from the lucerne plants more readily than usual during hot summer periods and during heavy thunder storms.

Considering the apparent effect of these environmental changes, an attempt was made to induce stressful states for insects kept in the laboratory and to record the influence of them, if any, on the wellbeing of caterpillars, larvae and pupae, their periods of survival and the incidence of latent or overt virus infection.

It is generally accepted that in a latent infection a state of equilibrium exists between the virus and the host (Smith, 1967). Latency may be due to a long association between host and virus (Cunningham, 1968) or to the presence of small amounts of virus (Steinhaus, 1949; Jaques, 1962; Smith, 1967). Latent infections are very common among insects and stress factors of various types, occurring naturally or experimentally produced, may change the situation from one of inapparent to apparent fatal infection causing an epizootic with high mortality (Aizawa, 1963; Smith, 1963).

A stress factor, or a stressor, is an environmental factor affecting the normal state of an animal. Stress is the reaction of the animal and one which may influence virus infection (Aruga, 1963). The stressor may either exalt a potential pathogen and induce a latent infection to become overt, as reported for cytoplasmic-polyhedroses (Tanada et al., 1964), nuclear-polyhedroses (Steinhaus, 1960b) and granuloses (Smith, 1967), or it may increase the virulence of a virus as reported for cytoplasmic-polyhedroses (Steinhaus, 1960b), nuclear-polyhedroses (Jaques, 1961) and granuloses (David and Gardiner, 1965). A stress factor may affect the susceptibility of the host by altering either the penetration rate of the virus or its replication rate, or both (Jaques, 1962; Smith, 1967). Doane(1969) suggested that rather than favouring the invasion of susceptible cells, a stressor interferes with the physiological processes that normally maintain the infection in the latent state. A stress condition might induce favourable conditions for the virus or those unfavourable for the host, or both.

The susceptibility of insects to different stressors varies according to the amount of virus present and the degree and duration of application of the stressor (Tanada and Chang, 1964). The genetic constitution of the species of a particular strain and the developmental stage are important in defining this susceptibility. The presence of modifiers that do not act as stress factors per se but make it easier for the stressor to extend its influence must also be considered (Aruga, 1963).

Environmental stress factors that exist in nature are

over-crowding, extremes of temperature and relative humidity, shaking by the wind and restriction on quantity or quality of food. Conditions in the laboratory may themselves cause stress (Steinhaus, 1949, 1960b; Thompson and Steinhaus, 1950; Smith, 1967; Cunningham, 1968). David et al. (1968) showed that in order to maintain a stock of insects carrying occult granulosis virus, the insects should be isolated, fed well and reared at low population density under conditions of moderate humidity, constant temperature and controlled light. Failure to provide these conditions might initiate stress factors that could trigger the activation of occult virus and the onset of disease. Even despite the care taken, spontaneous infections sometimes occurred without an obvious stressful state.

Over-crowding of the host species in nature is known to be associated with outbreaks of epizootic infections (Smit, 1936; Wilcox et al., 1956; Tanada, 1961; Smith, 1967). An induced infection of nuclear-polyhedrosis virus in a dense population of the gypsy moth in nature was reported by Doane (1970). In captivity, over-crowding was found to activate latent polyhedrosis and granulosis virus infections of various Lepidopterous insects (Aruga, 1963).

Epizootics are also common during hot and humid months (Michelbacher and Smith, 1943). When the insect population becomes dense and active, intensification of the disease occurs (Steinhaus, 1949; Thompson and Steinhaus, 1950).

The role of extremes of temperature, within the limits

of virus and host survival, in activating a latent infection has been studied in the laboratory and has given conflicting results. Aruga (1963) reported that the exposure of silkworms to the stress of heat and cold activated latent nuclear and cytoplasmic-polyhedrosis virus infections, while Steinhaus and Dineen (1960), Steinhaus (1960b) and David and Gardiner (1965) found that such extremes had no influence on inducing outbreaks of diseases in captivity.

The effect of temperature on the virus infection is reported to hasten mortality of the alfalfa caterpillar, the bollworm and the cabbage looper rather than to increase the incidence of the disease (Thompson and Steinhaus, 1950; Ignoffo, 1966b; Canerday and Arant, 1968) though both processes are known to happen (Doane, 1967).

Humidity was reported to have no effect on mortality by Pimentel and Shapiro (1962). Doane (1969) showed that stocks of larvae collected from dry and wet areas were equally susceptible. However, humidity does seem to play a modifying role in combination with temperature since in most cases high temperature and high humidity increase the incidence of polyhedrosis and granulosis virus infections but high temperature, without humidity, may not (Aruga, 1963).

Extreme climatic conditions are known to play a part in the initiation of infection by causing the larvae to drop to the ground (Thompson and Steinhaus, 1950). As mentioned above, thunderstorms and the shaking of plants appear to have this effect on Colias electo. Jaques (1961) found that rearing the cabbage looper on a shaker increased the incidence of nuclear-polyhedrosis virus infection four-fold.

According to Jaques (1962), reduced consumption of food does not create stress. This fact was confirmed by Steinhaus (1960b) for cytoplasmic-polyhedrosis virus infection of Heliothis zea and by Steinhaus and Dineen (1960) for granulosis virus infection of the variegated cutworm. In another Lepidopterous species, Bucher and Harris (1968) found that starvation acts as a stressful state for inducement of cytoplasmic-polyhedrosis virus infection.

There have been many reports on the lethal effects of applying a suspension of Bacillus thuringiensis (Berliner) endospores to insect larvae. The mortality caused among some species has favoured its use in the field as a method of biological pest control. It was used for instance with apparent success against Colias electo in the Stellenbosch area in 1951-1952 (Bedford, 1961). The related species, Colias eurytheme, (Stern et al., 1959) and the bollworm, Heliothis zea, (Tanada and Reiner, 1962) were also successfully controlled by the endospores preparation in America. According to H. Geertsema (Dept. Forestry, Cape Town, personal communication), it was not successful in increasing the mortality rate among larvae of the pine emperor moth, Nudaurelia cytherea capensis, in some South African forests but in other experimental areas the mortality of the same species varied from 30-80%. In laboratory investigations it was found that a high mortality rate and abundant nonoccluded virus were present in populations subjected to spraying but not in those control populations that were not sprayed with suspensions of

Bacillus thuringiensis. This suggests that the bacterial endospores preparation acted as a stressful condition in activating a latent nonoccluded virus infection.

An investigation was undertaken to ascertain whether shaking, spraying with a Bacillus thuringiensis preparation and exposure to raised temperature acted as stressors favouring virus infection. A comparison was made of these stressors and their ability to influence the incidence of nuclear-polyhedrosis virus infection and the time of death of the insects.

Another stress factor, injection of a foreign virus to activate a latent infection, was studied. In the present study, when the nuclear-polyhedrosis virus of Colias electo was administered to the silkworm larvae, Bombyx mori, a latent cytoplasmic-polyhedrosis virus infection was provoked. Free virus particles were injected into the silkworm haemocoel to avoid inactivation by antiviral substance in the gut juice (Aizawa, 1962) and because some silkworm strains have an inherited resistance to peroral infection (Watanabe, 1967).

Smith (1967) pointed out that in many instances, the inoculation of a foreign virus is a successful method of exalting a latent native virus infection. The activation of a cytoplasmic-polyhedrosis virus by a nuclear-polyhedrosis virus was observed by Xeros (1952), Smith and Rivers (1956) and Smith (1963). Tanada et al. (1964) reported the activation of a cytoplasmic-polyhedrosis virus by a granulosis virus.

A wide range of chemicals like EDTA, ether and formalin have been investigated as stressors (Steinhaus and Dineen, 1960; Aruga, 1963) as well as ultraviolet light (Pimentel and Shapiro, 1962; Steinhaus and Dineen, 1960) but conflicting results have been obtained. These agents were not used in the present study.

3.2. MATERIALS AND METHODS

3.2.1. Colias electo larvae from a stock collected at Elsenburg in November, 1969, were used. All instars were reared on fresh lucerne leaves in four groups of 40 larvae and each group kept in a glass jar, 15 cm diameter and 7.5 cm high. One group was used as a control; of the other three, each group received a different stress treatment carried out continuously for 72 hours. The lucerne was replenished twice a day.

A. One group of Colias electo larvae was reared at room temperature, 15-22°C, and was subjected to shaking. The shaking was produced by a rotating wooden platform attached to the motor of an automatic pipetting machine (Brewer). The glass container with the lucerne larvae was mounted on the platform and moved through a 5 cm radius at a rate of 92 cycles/min.

B. Another group of Colias electo larvae was reared at room temperature on lucerne leaves sprayed with Bacillus thuringiensis (Berliner) using Thuricide - a commercial

product of International Minerals and Chemical Corporation, U.S.A., containing 3 x 10^{10} viable spores per gram. An atomizer was used to spray the lucerne leaves with 15 ml of Thuricide diluted to give approximately 18 x 10^{8} spores/ml.

C. This group of larvae of Colias electo was reared in a 37°C incubator. A filter paper dipped in water was placed in the jar and wetted twice daily.

D. A control group of Colias electo larvae was reared at room temperature under 'normal working laboratory' conditions.

The mortality of the caterpillars was checked each day and the dead ones were removed. The presence of polyhedra in the dead larvae was determined by light microscopy.

3.2.2. A second batch of Colias electo caterpillars collected at Elsenburg in December, 1969, was used. Seventeen larvae of four different instars (2-5) were reared in a 37°C incubator for 72 hr. The same number of larvae of each instar larvae were reared at room temperature as a control. Both groups were fed fresh lucerne leaves and the food replaced twice daily. Mortality was checked every day and the dead larvae were removed. The dead larvae were allowed to decompose individually at room temperature for 3 days in universal bottles to which 1 ml of distilled water was added. The dead larvae were then cut into small pieces to allow most of the contained polyhedra to settle at the

bottom of the glass containers. The presence of inclusion bodies in 1 ml of the suspension was established by light microscopy and counts were made in a haemocytometer.

3.2.3. Silkworms were obtained by hatching eggs artificially out of season. The eggs from the previous season which had been kept at room temperature were transferred to 4°C for 40 days. They were then treated with 1% formalin for 2 min followed by immersion in 6N HCl at 45°C for 5 min. After a wash under tap water for 10 min the eggs were dried on a filter paper and left at room temperature (Nayar and Fraenkel, 1963). Most of the eggs hatched after 24 days. The larvae were reared in glass jars, 30 cm diameter x 12 cm high with not more than 50 larvae per jar and were fed on fresh mulberry leaves.

A volume of 0.05 ml of a saline suspension of the nuclear-polyhedrosis virus of Colias electo, released from 20 mg polyhedral preparation (4.2.7.), was administered to all instars by injection into the haemocoel. The control group received 0.05 ml sterile normal saline. 100 IU of penicillin and 100 µg of streptomycin per 1 ml were added to each inoculum.

The larvae were observed until they had died or until the pupae or the moths derived from them had died. The symptoms of infection were recorded. To determine the cause of death, smears of a drop of haemolymph from the dead larvae, pupae and moths were checked under the light microscope for the presence of polyhedra and the number of

polyhedra per drop of haemolymph (0.05 ml) was estimated.

Haemolymph of a few heavily infected caterpillars was centrifuged at 10,000 rpm for 10 min (rotor 40 Spinco model L ultracentrifuge) to yield a pellet of polyhedra. The supernatant fluid was then centrifuged at 30,000 rpm for 30 min to obtain free virus particles in the pellet. The two pellets thus obtained were studied by electron microscopy.

To study the effect of the stress on the succeeding generation, ten newly and artificially hatched larvae were checked for the presence of cytoplasmic-polyhedral inclusion bodies in their body fluids. They were crushed on a microscope slide in a drop of water and were examined under the light and the electron microscopes.

3.3. RESULTS

3.3.1. The effect of different conditions of stress on the incidence of the nuclear-polyhedrosis virus infection of Colias electo and on the time of death.

A comparison was made between the incidence of fatal virus infection of Colias electo larvae and pupae in the three groups subjected to stressful conditions and a control group maintained under ordinary laboratory conditions. These insects were collected in November, 1969. The surviving larvae and pupae which were normal in appearance were found to contain no polyhedra. In those insects which died, with the exception of three, polyhedra were seen. The mortality apparently due to the virus infection is summarized in Table 3.3.1.

Table 3.3.1. Mortality of Colias electo larvae and pupae with nuclear-polyhedrosis virus infection at intervals after subjection to different conditions of stress.

Stress	No. of larvae	Day of observation after treatment	Mortality						Survival	
			Larvae		Pupae		Total (larvae and pupae)			
			No.	% of treated larvae	No.	% of treated larvae	No.	% of treated larvae	No.	% of treated larvae
Shaking	40	1 2 3	0 2 0	0 5.0 0	2 0 0	5.0 0 0	2 2 0	5.0 5.0 0	38 36 36	95.0 90.0 90.0
	Total		2	5.0	2	5.0	4	10.0		
Spraying with Endospores	40	1 2 3	10 0 4	25.0 0 10.0	1 0 0	2.5 0 0	11 0 4	27.5 0 10.0	29 29 25	72.5 72.5 62.5
	Total		14	35.0	1	2.5	15	37.5		
Incubation at 37°C	40	1 2 3	17 3 1	42.5 7.5 2.5	7 0 0	17.5 0 0	24 3 1	60.0 7.5 2.5	16 13 12	40.0 32.5 30.0
	Total		21	52.5	7	17.5	28	70.0		
Nil (Controls)	40	1 2 3	0 1 0	0 2.5 0	3 0 0	7.5 0 0	3 1 0	7.5 2.5 0	37 36 36	92.5 90.0 90.0
	Total		1	2.5	3	7.5	4	10.0		

The results of Table 3.3.1. are presented diagrammatically in Figs. 10 and 11 as the November batch.

It will be seen from Table 3.3.1. that:

i. shaking produced by a rotary movement of 92 cycles/min through a 5 cm radius caused no greater mortality than occurred among the larvae in the control group of similar size.

ii. the effect of spraying the leaves on which the larvae were feeding with a suspension of endospores of Bacillus thuringiensis had an appreciable effect on the mortality rate. Within 3 days, 37.5% of the larvae and pupae had died, while only 10% of the controls had succumbed.

iii. the high mortality rate associated with incubation of the larvae and pupae at 37°C is very striking. Within 3 days 70% of the test insects had died compared with only 10% in the control group.

In order to test the validity of this observation the experiment was repeated with larvae of Colias electo collected in December, 1969. The mortality of this batch is summarized in Table 3.3.2.

3.3.2. The effect of raised temperature on the incidence of infection, on the time of death and on the concentration of nuclear-polyhedrosis virus in different larval stages of Colias electo.

The larvae for this experiment were collected in December. All of those held at 37°C in the incubator, died

Fig. 10. The effect of three stressful conditions on the incidence of nuclear-polyhedrosis virus infection of Colias electo larvae and pupae.

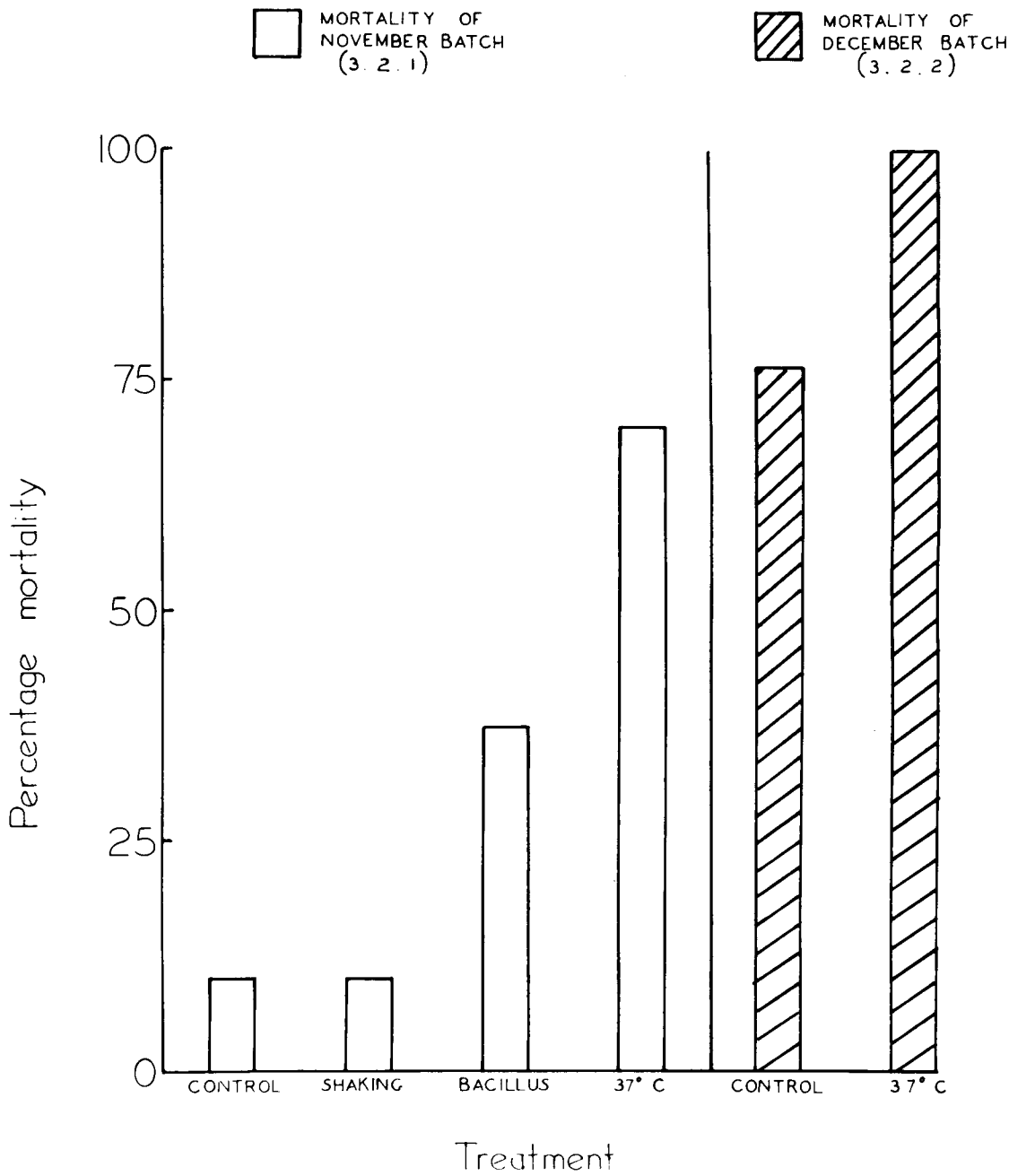
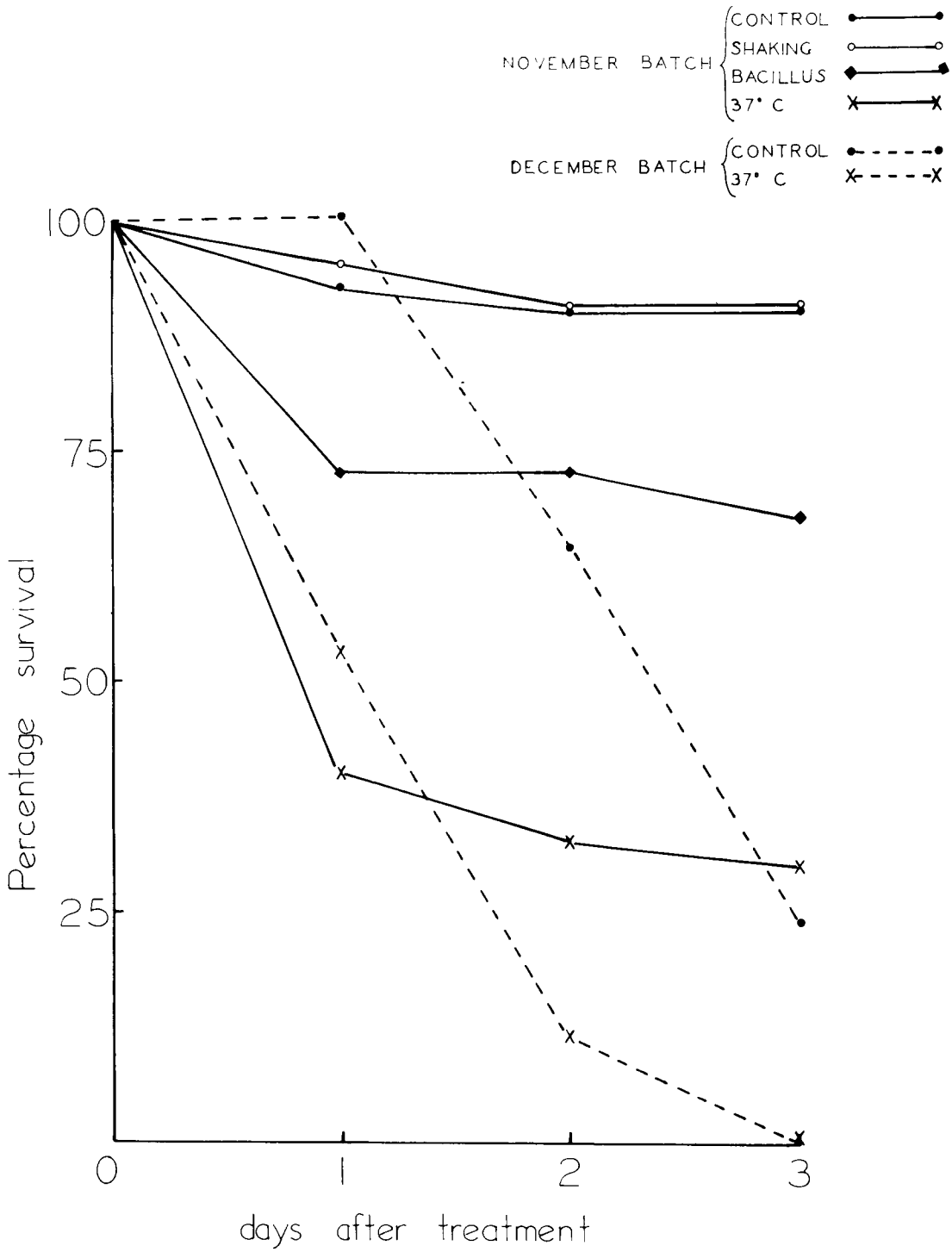

MORTALITY OF NOVEMBER BATCH (3.2.1)
MORTALITY OF DECEMBER BATCH (3.2.2)
100
75
50
25
0
Percentage mortality
CONTROL
SHAKING
BACILLUS
37° C
CONTROL
37° C
Treatment

Fig. 11. The effect of three stressful conditions on time of death of Colias electo larvae and pupae caused by the nuclear-polyhedrosis virus.


NOVEMBER BATCH
CONTROL
SHAKING
BACILLUS
37° C
DECEMBER BATCH
CONTROL
37° C
100
75
50
25
Percentage survival
0
1
2
3
days after treatment

within 3 days. The control larvae showed a much higher mortality (76.5%) than in the previous experiment with larvae collected in November. The difference between the treated and the control groups, however, is still significant. The results are presented in Table 3.3.2. An additional difference is apparent in that almost half of the test larvae died on the first day, whereas in the control group the larvae survived the first day and died only on the second and third days.

Table 3.3.2. Mortality of Colias electo larvae with nuclear-polyhedrosis virus infection at intervals after treatment.

Treatment	No. of treated larvae	Day of observation after treatment	Mortality		Survival	
			No.	% of treated larvae	No.	% of treated larvae
37°C	17	1 2 3	8 7 2	47.0 41.2 11.8	9 2 0	53.0 11.8 0
	Total		17	100.0		
Room temperature	17	1 2 3	0 6 7	0 35.3 41.2	17 11 4	100.0 64.7 23.5
	Total		13	76.5		

The results of Table 3.3.2. are presented diagrammatically in Figs. 10 and 11 as the December batch.

Data on the concentration of nuclear-polyhedra in the dead larvae are listed in Table 3.3.3. Enumeration of polyhedra per ml in suspension of the dead larvae showed that the virus concentration was remarkably similar in the test

Table 3.3.3. Number of polyhedra/ml per dead larva exposed to 37°C for 3 days in different larval instars at intervals after treatment.

Larval instar	Day of observation after treatment	No. of polyhedra per ml at 37°C	No. of polyhedra per ml at room temperature (controls)
2	1	8×10^7	
2	1	18×10^7	
2	3	106×10^7	117×10^7
2	3		45×10^7
Mean values		44×10^7	81×10^7
3	1	20×10^7	
3	2		16×10^7
3	3	52×10^7	14×10^7
Mean values		36×10^7	15×10^7
4	2	98×10^7	13×10^7
4	2	36×10^7	48×10^7
4	3		124×10^7
Mean values		67×10^7	62×10^7
5	2	129×10^7	116×10^7
5	2	125×10^7	
5	3		146×10^7
Mean values		127×10^7	131×10^7
Total mean values		69×10^7	72×10^7

and the control larvae. The variation in concentration between days 1, 2 and 3 is not consistent or significant. There is a suggestion that the virus concentration in the second and third instars is lower than that in the fourth and fifth instars but these differences too were not significant.

This experiment confirmed to some extent the observation that raised temperature increased the rate and the total mortality of the larvae over the control group, but there is no strong evidence that there is a difference in susceptibility of the various larval stages and furthermore there is no significant difference in the final concentration of virus in the dead larvae from the test and control groups.

3.3.3. The effect of the injection of nuclear-polyhedrosis virus of Colias electo on the mortality rate and the occurrence of cytoplasmic-polyhedrosis virus infection in the silkworm, Bombyx mori.

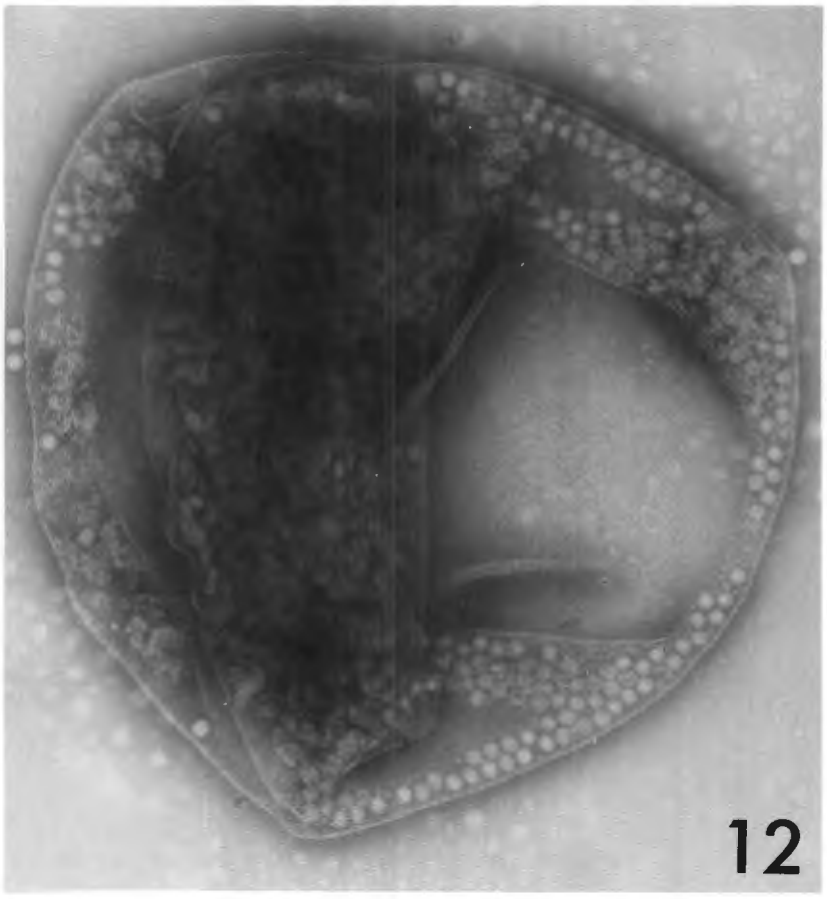
Seventy-three caterpillars of Bombyx mori were inoculated with a fixed dose of Colias electo nuclear-polyhedrosis virus particles and a control group of 109 larvae were given an injection of sterile normal saline of the same volume.

The cytoplasmic-polyhedra of Bombyx mori were easily distinguishable from the nuclear-polyhedra of Colias electo by electron microscopy. In the silkworm the cytoplasmic inclusion body was approximately 1 μ in diameter (Fig. 12) and the occluded virus particles were spherical in shape with a diameter of 22-25 nm (Fig. 13). By contrast, the

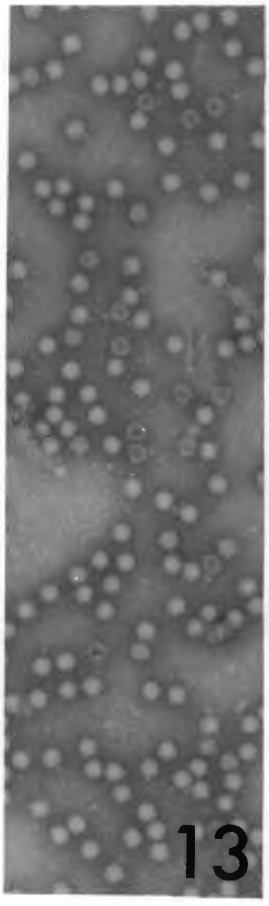
Fig. 12. Electron micrograph of an intact silkworm cytoplasmic-polyhedron occluding the spherical virus particles. Negatively stained. Magnification X 100,000.

Fig. 13. Electron micrograph of free cytoplasmic-polyhedrosis virus particles of the silkworm. Negatively stained. Magnification X 100,000.

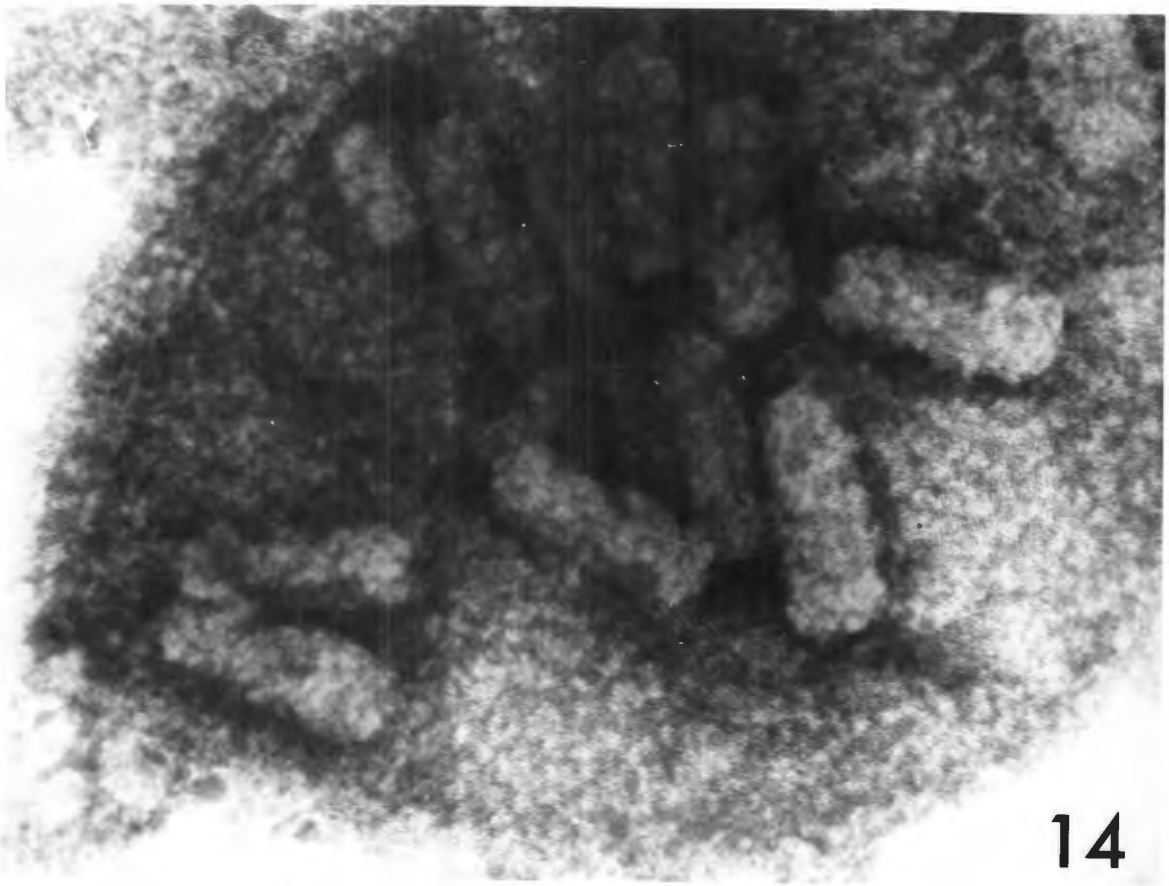
Fig. 14. Electron micrograph of a nuclear-polyhedron of Colias electo occluding rod-shaped virus particles. Negatively stained. Note the larger size of the polyhedron as compared to that in Fig. 12. Magnification X 100,000.



12



13



14

average nuclear-polyhedra of Colias electo were larger and contained rod-shaped virus particles (Fig. 14). However, the nuclear-polyhedra were not recovered from the silkworms injected with virus due to the small dose inoculated.

Observations were made on the mortality rate and the presence of cytoplasmic-polyhedra in the larvae, pupae and moths of Bombyx mori following injection of 73 larvae with nuclear-polyhedrosis virus of Colias electo compared with 109 control larvae injected with saline. These are listed in Tables 3.3.4. and 3.3.5.

Table 3.3.4. The effect of injection of silkworm larvae with the nuclear-polyhedrosis virus of Colias electo on the mortality rate in various developmental stages.

Developmental stage in which death occurred		No. and % of dead insects (% of injected larvae)	
		No.	%
Larvae	Injected with virus	7	9.6
	Injected with saline (control)	0	0
Pupae	Developed from larvae injected with virus	17	23.2
	Developed from control larvae	7	6.4
Moths	Developed from larvae injected with virus	49	67.1 *
	Developed from control larvae	102	93.5

* The high figures are also due to natural termination of the life cycle.

From Table 3.3.4. it can be seen that there is a difference in the mortality rate between the different developmental stages, both of the controls and the group subjected to injection of virus during the larval stage.

Of the larvae injected with virus, 9.6% died at this stage and all were found to contain cytoplasmic-polyhedra. No deaths occurred among the control larvae.

Among the pupae which developed from the surviving larvae, there was again a significantly higher mortality in the group injected with virus (23.2%) compared with that in the control group (6.4%) and, furthermore, while polyhedra could be found in 90% of the former, they were present in only 50% of the latter.

Most of the larvae in this experiment reached the adult stage and died as moths. It was difficult to determine whether the deaths of the moths resulted from natural causes or were due to the cytoplasmic-polyhedrosis virus infection. However, the occurrence of the polyhedra, whether in lethal or sublethal dose, was observed in 90% of the moths of the test group as compared with only 50% of the control group.

The additive results of the occurrence of cytoplasmic-polyhedra in the larvae, pupae and moths that died are presented in Table 3.3.5.

Table 3.3.5. The effect of injection of silkworm larvae with the nuclear-polyhedrosis virus of Colias electo on the concentration of cytoplasmic-polyhedra in all the developmental stages.

Treatment	No. and % of insects containing more than 20 polyhedra per drop of haemolymph		No. and % of insects containing 1-20 polyhedra per drop of haemolymph		No. and % of insects containing no polyhedra	
	No.	%	No.	%	No.	%
Larvae injected with virus	57	78.0	9	12.3	7	9.5
Larvae injected with saline (control)	10	9.2	43	39.4	56	51.3

The results of Table 3.3.5. are presented diagramatically in Fig. 15 to show the wide divergence of polyhedral concentration of the two groups of insects.

While approximately 50% (51.3%) of the silkworms which acted as controls had no polyhedra, only a few were heavily infected with the cytoplasmic-polyhedra (9.2%). The larvae injected with virus and the insects deriving from them showed a contrary pattern in that most of them were heavily infected (78%) and only 9.5% had no detectable polyhedra.

Silkworms with normal appearance which were either noninfected or inapparently infected were compared with those with overt disease (Fig. 16). The moths with no polyhedra and those with 1-20 polyhedra per drop of haemolymph were of normal appearance and laid fertile eggs.

Fig. 15. The effect of injection of nuclear-polyhedrosis virus of Colias electo on the occurrence of cytoplasmic-polyhedrosis virus infection of Bombyx mori.

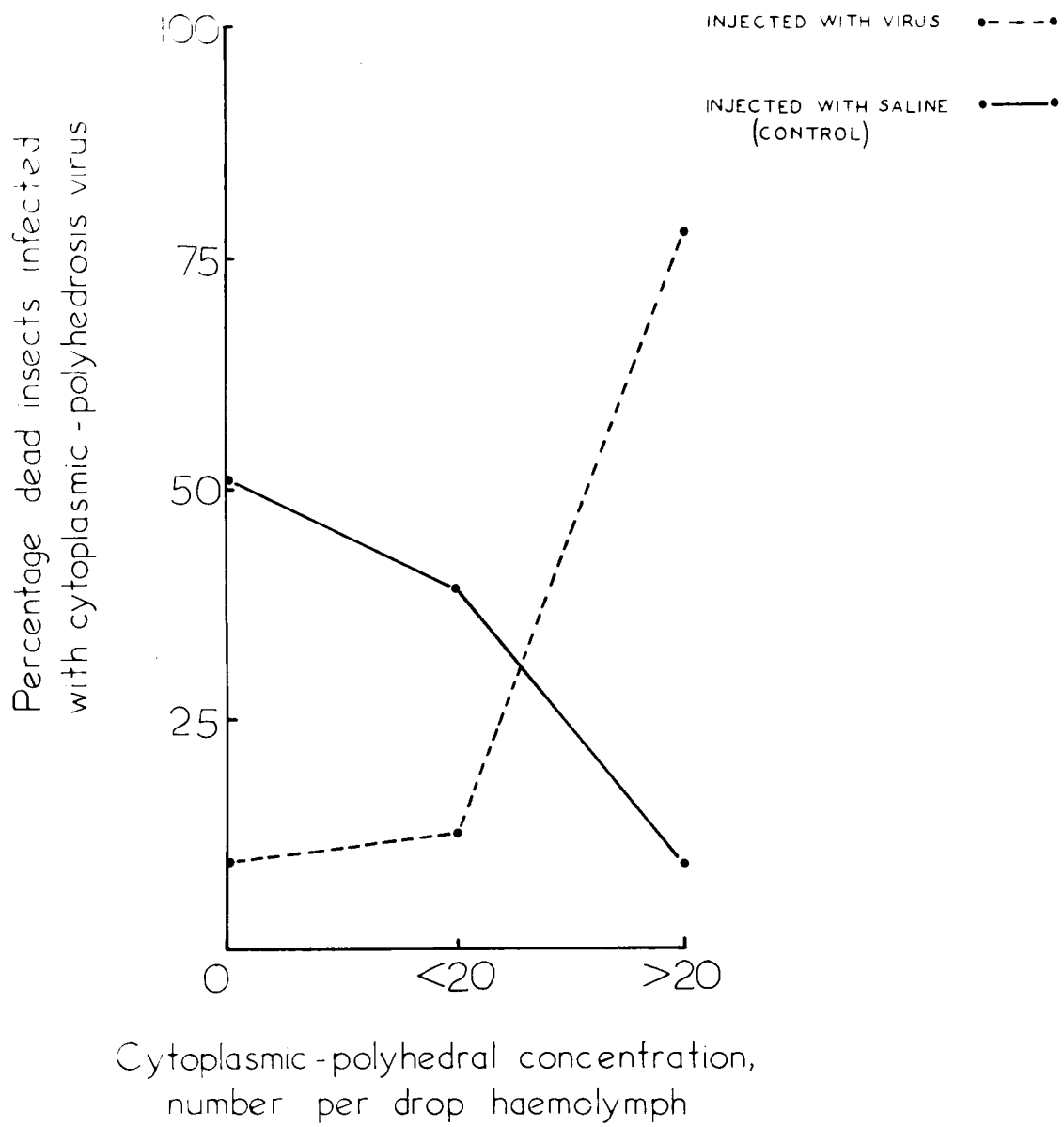
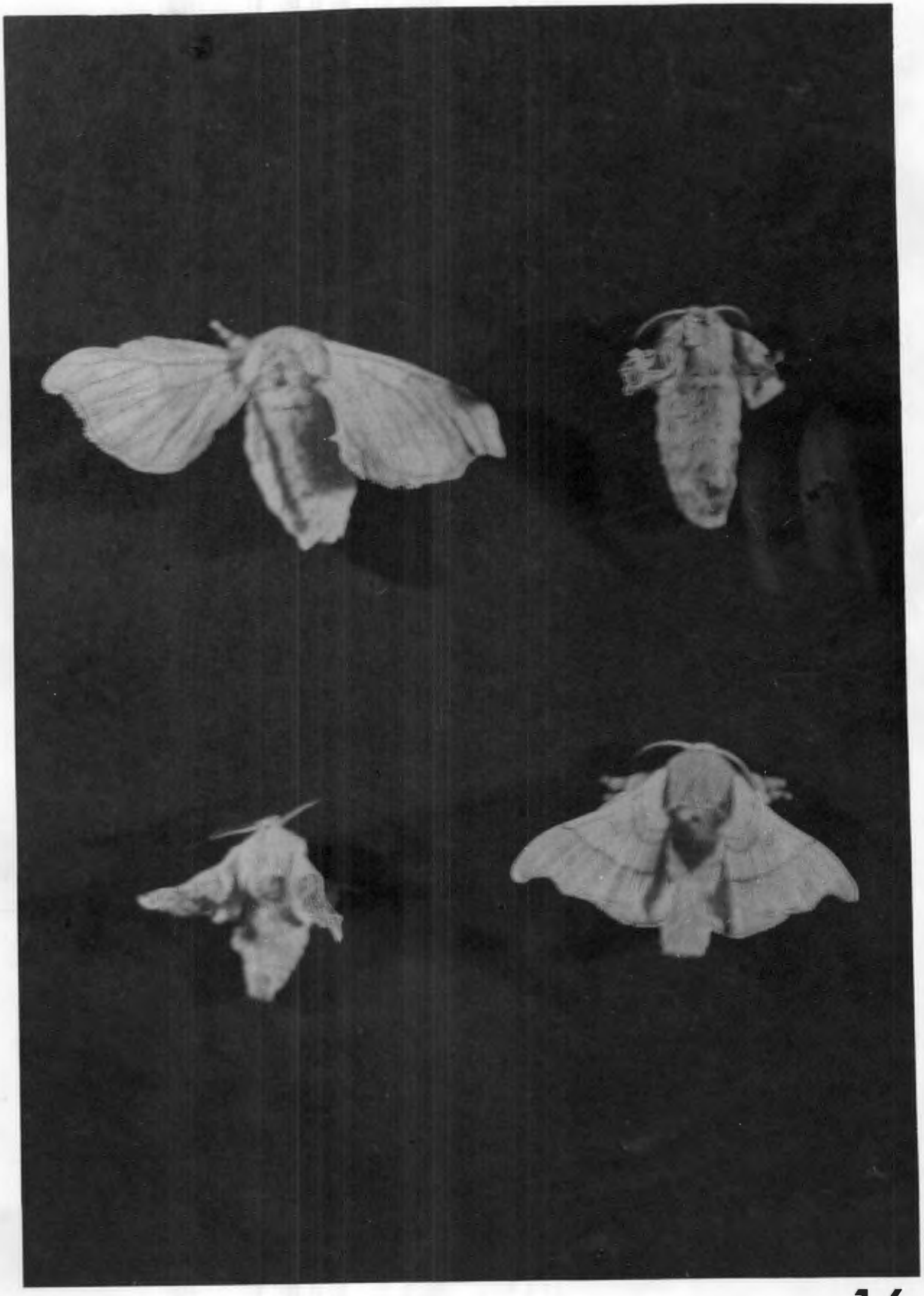

INJECTED WITH VIRUS
INJECTED WITH SALINE (CONTROL)
Percentage dead insects infected with cytoplasmic-polyhedrosis virus
100
75
50
25
0
<20
>20
Cytoplasmic-polyhedral concentration, number per drop haemolymph

Fig. 16. The appearance of silkworm moths, Bombyx mori, derived from larvae which had been injected with the nuclear-polyhedrosis virus of Colias electo. Control larvae received only sterile saline.

Upper left and lower right: moths with normal appearance from the control larvae injected with sterile saline.

Upper right and lower left: moths with abnormally small bodies and with grossly underdeveloped and malformed wings resulting from the activation of a latent cytoplasmic-polyhedrosis virus infection of the silkworm following the injection of a nuclear-polyhedrosis virus of the lucerne caterpillar, Colias electo.



The haemolymph was clear in the insects with no polyhedra and light brown in those that had 1-20 polyhedra per drop. The heavily infected insects (those with more than 20 polyhedra per drop) had a dark brown haemolymph and many of the moths had an abnormally small body with underdeveloped wings. Not all of the infected moths laid eggs but those that did produced eggs that were often infertile.

Out of ten newly hatched larvae of the second generation after treatment, nine had cytoplasmic-polyhedral inclusion bodies. Most of the stock, nevertheless, managed to complete the life cycle, suggesting that the virus infection was a latent one.

3.4. DISCUSSION

Of the stressful states produced in the laboratory, only incubation at 37°C and the injection of a foreign virus produced results that were significant.

The effect of shaking produced by a rotary movement caused no greater mortality than that seen in the control insects. Jaques (1961) used 150 cycles/min through a 2.5 cm radius and thereby increased the mortality of the cabbage looper larvae from a nuclear-polyhedrosis virus infection, but in the present experiments 92 cycles/min through a 5 cm radius were ineffective in influencing the mortality rate.

A higher percentage mortality occurred in the group of larvae fed lucerne leaves which had been sprayed with

Bacillus thuringiensis than what occurred in the control untreated group. Since the bacterial suspension is known to possess a toxin for the larvae it was difficult to be certain whether the increased mortality was due wholly or partly to the toxicity. Experiments with higher concentrations of bacterial endospores were not attempted since the dose used in the present experiments exceeded by a factor of two the dose used with apparent success in the field in efforts to control the pests by biological control methods. Nevertheless, the spray of a combined preparation of Bacillus thuringiensis and a virus may well prove more efficient in the field than either one of them alone, as pointed out by Steinhaus (1951) and Stelzer (1965).

A very striking result was seen when subjecting the larvae to the stressful condition of increased temperature in the 37°C incubator.

In the first experiment with insects harvested in November, the survivors were reduced to 40% on the first day and to 30% by the third day amongst those exposed to 37°C. This must be compared with a survival of 90% among the control group kept at room temperature.

In the second experiment with insects collected in December, despite the significantly higher mortality (76.5%) among the controls after three days, there were no survivors among the incubated larvae.

Both experiments show the higher mortality among the insects in the incubator over those on the laboratory bench with an earlier onset of illness and death in the former than in the latter. The differences in the mortality rates of

the control groups in the two experiments may well be an expression of the higher incidence of nuclear-polyhedrosis virus infections among the larvae collected in the appreciably hotter month of December.

The various larval stages were all susceptible to the effect of raised temperature in bringing about an increased mortality from virus infection. The results of counting the polyhedra in the dead insects each day showed that the virus concentrations in those kept at 37°C were not significantly different from those held at room temperature. The samples of each larval instar for each day of the experiment were too small for statistical analysis. However, the total mean values of polyhedra per ml suspension showed no significant differences. The inference from this is that the raised temperature resulted in a larger proportion of the larvae achieving a fatal concentration of virus in a slightly shorter time than in the control larvae that died.

It is difficult to assess the relative contributions of these patently artificial conditions of stress applied in the laboratory since they are clearly not the only factors involved.

Harvesting, transporting and handling the insects all constitute stressful conditions. Shaking the plants and gathering those caterpillars that had fallen to the ground must also be regarded as traumatic and stressful.

This background of new and artificial experiences for the insects may well be enough to trigger the processes leading to death of the insects in the control group in the

laboratory, but these conditions were common to the test and control insects alike. If these conditions were responsible for the activation of occult virus to produce lethal virus infections in the control groups, it is presumed that the added stresses of raised temperature or injection of a foreign virus increased the rate and the extent to which this occurred.

Since it is well known that deaths among Colias electo caterpillars from virus infection occur in nature, it is perhaps not surprising that the abnormal environmental factors such as high temperature, high humidity, thunder clap and perhaps even the sound of the farmer cracking his whip in the lucerne fields, may contribute to the observed higher mortality under these conditions.

Care was exercised in the laboratory to reduce extraneous factors to a minimum and to ensure that as far as possible similar conditions were maintained for both the control insects and the treated insects. Thus the quality and quantity of the food as well as the population density were controlled. Virus transmission by contact was limited by removing the dead caterpillars and replacing the food contaminated with discharged haemolymph of sick and dead larvae twice daily. All larval instars were used in all the present experiments to eliminate the influence of age on incidence of infections (Stairs, 1965), on the time of death (Ignoffo, 1966a) and on the susceptibility to stressors (Aruga, 1963; Bucher and Harris, 1968).

A control of the humidity was also thought to be necessary since at 37°C evaporation from the leaves and the larvae is greater than at room temperature. Therefore,

damp filter paper was placed twice a day in the jar with the insects in the incubator. Later, when all collected lucerne caterpillars were routinely incubated at 37°C to increase the virus yield, no similar attempts were made to increase the humidity and the mortality rate remained as high and appeared as early. It is therefore suggested that humidity plays no part as a stressor as supported by Thompson and Steinhaus (1950) who worked with the related species, Colias eurytheme.

Two factors, however, were not controlled. They are the transfer of larvae from the laboratory to the incubator (Aruga, 1963) and the lack of light in the incubator (Smirnoff, 1967). It was possible that 37°C for 72 hr inactivated the virus or adversely affected the caterpillars but the fact that a certain percentage of the population kept in the incubator developed normally and did not succumb to the disease makes this suggestion unlikely. This finding is in accordance with Tanada and Chang (1968) and Thompson (1959) who found that a temperature of the order of 37°C can interfere with invading virus, but once the virus had entered, neither the virus nor the insect are affected.

The other stressor to yield a significant result was the injection of a foreign virus into the larvae of Bombyx mori. Here the injection of a foreign rod-shaped nuclear-polyhedrosis virus into the silkworm resulted in the activation of a latent spherical cytoplasmic-polyhedrosis virus infection, a phenomenon known to occur in many other insect species (Smith, 1967).

The activated virus was apparently fatal, since the larvae and most of the pupae that died had more than 20 polyhedra per drop of haemolymph. Among the moths it is not known whether the 90% with virus died from the infection or died naturally, carrying an induced sublethal dose. The small body size, malformed wings and reduced fertility which were observed in many heavily infected moths are typical signs caused by sublethal infection as discussed by Tanada and Tanabe (1964) and Neilsen (1965). Similar findings were recorded with some Colias electo butterflies (2.3.1.).

It can be stated that the injection of foreign virus into the larvae changed the mortality rate of larvae and pupae. It clearly altered the occurrence of the overt infection in the population and the concentration of polyhedra in individual hosts of all developmental stages, including the moths. It was demonstrated that in the control group 40-50% of the population had the virus in an occult state. The resistant portion of the population may be represented by the 9.5% of the group injected with virus containing no polyhedra. The fact that most of the group injected with virus had a higher concentration of inclusion bodies than the control group, favours the idea that a latent infection is associated with a low concentration of virus which is either undetectable or low enough (1-20 polyhedra per drop) not to cause any external symptom or death. The 9.2% of the heavily infected control group (more than 20 polyhedra per drop) may represent the more sensitive portion of the population. It is, however, possible that this group, together with the control insects containing the

low concentration of polyhedra (1-20 per drop), are those which suffered from other stress factors such as the trauma resulting from the injection, other laboratory manipulations or new circumstances foreign to the insects. These conditions might have also increased the incidence of the infection induced by the foreign virus.

The examination of the succeeding generation showed that the stress of injecting a foreign virus which induced a latent infection in one generation, did not have the same effect on the next generation. Similar results were obtained by David and Gardiner (1965) with crowding as a stressful condition which enhanced a granulosis virus infection in one generation only. Although in a latent state, it was shown that the cytoplasmic-polyhedrosis virus was transmitted transovarially through the interior of the egg to the offspring. This was proved by disinfection of the surface of the eggs during the hatching procedure (3.2.3.). This association between a latent infection and transovarial transmission is a known phenomenon described by Hukuhara (1962), Tanada (1962) and Smith (1967).

In the present work it was possible to distinguish between the foreign and the native induced viruses by morphology. It is suggested that in cross transmission studies, care should be taken to ensure that activation of a native virus had not taken place.

The discovery of activation of a native virus by a foreign virus may be of economic importance since despite the specificity of a virus it may still be used to activate viruses of other hosts. Since Colias electo and Heliothis

armigera are known to be present together with other insect pests in lucerne fields in South Africa (Smit, 1936), it might be of value to carry out cross activation studies with the viruses of these insects.

CHAPTER 4

ISOLATION AND PURIFICATION OF THE INCLUSION BODIES AND THE VIRUS PARTICLES

4.1. INTRODUCTION

In procedures for isolating and purifying occluded viruses, two steps are involved. The first step is isolation and purification of the inclusion bodies, the second is the liberation of the virus particles from the inclusion bodies and their purification. In each it is important to be able to disperse any aggregates which may form.

4.1.1. Isolation and purification of the INCLUSION BODIES.

A. Isolation by gravity-settling. This procedure is based primarily on the insolubility of the nuclear-polyhedra and the granulosis capsules in water (Bolle, 1873, in Bergold, 1963a). Mechanically fragmented infected dead insects are suspended in water. The inclusion bodies are released from the infected tissues through autolysis and bacterial putrefaction but are themselves not affected in the process. This resistance to decomposition by bacteria and fungi is mentioned by Jaques and Huston (1969); resistance to the host's common proteolytic enzymes is recorded by Bergold (1953). The initial separation of the inclusion bodies

from debris takes place by gravity-settling when the inclusion bodies sink and much of the insect debris floats. The whole process depends on allowing sufficient time for the viral inclusions to be released from the decomposed tissues but not enough to favour bacterial overgrowth.

B. Coarse filtration. Debris and large non-viral particles that have settled together with the inclusion bodies may be removed by coarse filtration.

C. Differential centrifugation. Residual debris, large bacteria and sedimentable particles in the water are separated from the inclusion bodies by this process. Unfortunately, many bacteria are of a size similar to that of the inclusion bodies and have to be removed by other procedures. Loss of inclusion bodies at this stage of centrifugation is mainly due to the formation of aggregates, hence there is a limit to the number of times this process may be repeated before submitting the aggregates to the treatments that follow.

D. Water-organic solvent phase system. This procedure is based on a selective action of organic solvents on the inclusion bodies and the non-viral components, mainly bacteria and cellular debris. The mechanism of this action depends either on lipid extraction or on surface inactivation of proteins, or on both (Philipson, 1967). While lipids are an integral part of the walls of bacterial and insect cells, the inclusion bodies are 95% protein and

contain no lipids (Bolle, 1874, in Smith, 1967). Thus, the inclusion bodies remain in the aqueous phase, while the lipid-containing structures appear in the organic solvent phase.

When surface inactivation of proteins takes place it is due to the strong tension which exists between the two phases. The resultant denatured proteins form a layer at the interface, leaving the inclusion bodies in the watery phase which can then be extracted.

Ether, methanol, ethanol, chloroform, butanol and various combinations of these organic solvents have been used for isolating virus particles from tissues. Gessler et al. (1956a, b) first proposed the use of halogenated fluorocarbons for isolating virus particles from tissues. Bergold (1959) introduced the use of trichlorotrifluoroethane, $CF_2Cl-CCl_2F$, for the separation of insect virus inclusion bodies from contaminating substances. According to Philipson (1967), the actual basis of selectivity is not known and contradictory reports are found in the literature on fluorocarbon treatment. Most authors agree that fluorocarbon acts as a mild lipid solvent since most lipid-containing viruses are disrupted by it; some, however, are not. It also denatures proteins but having a low interfacial tension (1.5 dynes/cm) it is not very efficient for this purpose. Philipson (1967) and Faulkner et al. (1961) are of the opinion that, in some instances, the organic solvent selectively denatures proteins according to differences in their size. However, Gessler et al. (1956b) suggest that large particles such as bacteria and aggregates

of viruses, are broken up by the 'emulsification' during shaking with fluorocarbon while the separation of viral and non-viral proteins occurs according to the different 'wetting power' or attraction of the water and the fluorocarbon on the treated material.

The advantages of the now widely used fluorocarbon solvent are its chemical inertness and relative non-toxicity. Its low solubility in water, compared with other organic solvents (less than 0.005% at 25°C) enables a quick separation of the water and the fluorocarbon phases without losing material which otherwise could have sunk to the interface. As the fluorocarbon is very volatile, a suspension of virus or protein may be freed of it in a very short time by allowing the organic solvent to evaporate under atmospheric conditions. Being a mild organic solvent, fluorocarbon removes only part of the non-viral impurities but at the same time is safe enough to preserve the integrity of the inclusion bodies. Whitcomb et al. (1968) describe inactivation of an insect virus by butanol and chloroform but not by fluorocarbon. The latter was even used for the purification of free cytoplasmic-polyhedrosis virus particles (Miyajima et al., 1969) but it has not been tried on free rod-shaped virus particles (nuclear-polyhedrosis and granulosis) since they may become inactivated. The outer membrane of these virus particles contains a layer of lipid susceptible to fluorocarbon even though surrounded by carbohydrates and proteins (Ponsen et al., 1965).

Because the basis of the selectivity governing the use of fluorocarbon is not known, the results generally cannot

be predicted. Therefore the purification procedure for each of the viruses and inclusion bodies has to be tested empirically to establish the requisite conditions.

E. Sonication. The disruption of bacteria and cellular debris, as well as the dissociation of inclusion body aggregates may be achieved by this method. It also proved to be most efficient in releasing the inclusion bodies in the commercial preparation of the nuclear-polyhedra of Heliothis zea which were trapped in the diluent powder.

Ultrasonic waves are sound waves with frequencies over 16 kc/sec (Blitz, 1967). When propagated in a liquid, they cause alternate positive and negative acoustic pressures which cause compression and expansion respectively. This results in a phenomenon known as cavitation which is responsible for disruption of cells and dissociation of aggregates (Peacocke and Pritchard, 1968).

When air is dissolved in liquid, gaseous cavitation occurs. This involves a spontaneous growth of unstable gas bubbles in regions of low pressure and a resolution of the gas from the bubbles in the liquid in the regions of high pressure. The bubbles then move to regions of lower sonic intensity and less cavitational density so that the bubbles usually escape to the surface. The high velocity of this movement of bubbles may degrade cells by friction.

When there is no dissolved air but only water vapour, a transient cavitation occurs. This results in the formation of bubbles that grow under expansion to form vapour filled cavities which collapse under contraction. The energy released with the collapse is enough to break the cell wall

either directly or by giving rise to shock waves moving at high speed.

Hughes and Nyborg (1962) claim that an additional eddying effect is caused by the sound waves passing through two phases of different acoustic absorbance. Thus a flow of liquid is caused around both the bubbles and the cells and also inside the cells. The circulation of the cell fluids can thus cause death without disruption even at lower amplitudes than those necessary to induce collapse of the bubbles.

The mechanism of cell disruption may involve any one of the three interpretations outlined above according to the amount of gas in the liquid, the intensity of the ultrasonic waves, the susceptibility of the cells and the environmental conditions. Because of the variables involved, the level of degradation of biological cells under sonication is also difficult to predict quantitatively and has to be employed empirically for each type of cell.

As reported by Bucher (1963) and Faust and Estes (1965), nuclear-polyhedra will withstand considerable intensity of ultrasonic treatment. The same applies to granulosis capsules (Faust and Adams, 1967). A commercial preparation of nuclear-polyhedra of Heliothis zea, similar to the one used in this study, was successfully purified by ultrasound (Estes and Faust, 1966).

After sonication, differential centrifugation may be used to separate the degraded particulate matter and residual aggregates from the singly dispersed inclusion bodies.

F. Lyophilization. Inclusion bodies may be stored in a freeze-dried state in sealed containers at 4°C. Nuclear-polyhedra are reported to be stable in this state for 20-37 years (Steinhaus, 1960a; Bergold, 1963a) and granulosis inclusion bodies up to 5 years (Huger, 1963). In this study lyophilization was achieved by forming a cold trap around the containers of the inclusion body suspension so that under vacuum the sublimed ice forms a condensate on the cold layer.

4.1.2. Isolation and purification of the VIRUS PARTICLES.

A. Liberation of the virus particles from their inclusion bodies.

As early as 1893, Bolle (Bergold, 1964) noticed that inclusion bodies would dissolve in weak alkali and strong acids. The same effect was noticed by Glasser and Chapman in 1916, but it was first achieved experimentally by Bergold in 1947 (Hughes, 1950). He developed a general technique of using sodium carbonate, Na_2CO_3, strong enough to overcome the considerable buffer capacity of the inclusion body protein on the one hand and on the other hand weak enough to avoid the destruction of the liberated virus particles (Bergold, 1953). The virus particles will be destroyed by a concentration of alkali only 30% higher than that needed for dissolution of the inclusion bodies (Bergold, 1964).

The mechanism of the digestion of the inclusion bodies by Na_2CO_3 in vitro was found to be due to the dissolving of the silicate content of the inclusion body. In this way, hydrophobic groups are removed and the polyhedra or granulosis

capsules dissolve, liberating their virus particles (Estes and Faust, 1966; Faust and Adams, 1966; Faust and Adams, 1967). These workers suggested that *in vivo* proteolytic action on the inclusion body protein occurs only after the primary action of the gut alkali.

Though the general digestion procedure is similar for all inclusion bodies, the molarity of the alkali, the duration of exposure to it, the temperature and the relative amounts of material and alkali should be determined for each virus.

B. Differential centrifugation. The degraded inclusion body protein, the free virus particles and the residual intact polyhedra or capsules differ in their mass and therefore are easily separated from each other by centrifugation. The lowest speed for the shortest time should be used to sediment the virus particles in order to preserve their integrity and activity.

4.2. MATERIALS AND METHODS

4.2.1. The viruses.

The same procedures for isolation and purification with small variations in detail were used for the nuclear-polyhedrosis virus of *Colias electo* and the granulosis virus of *Heliothis armigera* and their inclusion bodies.

The nuclear-polyhedra of *Heliothis zea* were supplied in powder form as described in 2.2.4. Single and aggregated polyhedra together with bacteria and insect debris were trapped in the powder matrix and selective methods for

removal of all the non-viral constituents had to be developed empirically. These methods are described separately.

4.2.2. The diluent.

Double distilled water free of CO_2 was used as diluent throughout.

4.2.3. Centrifugation.

All the centrifugation at low speed, up to 2,000 rpm, were carried out in 10 ml glass centrifuge tubes either in the MSE 'Magnum Refrigerated' centrifuge in a standard (8 places) swing-out head, or in an 'International' clinical centrifuge without cooling.

For higher speeds, 10 ml cellulose nitrate centrifuge tubes were used in a Spinco model L ultracentrifuge, No. 40 rotor, at 4°C.

4.2.4. Observations.

The purity and recovery of the virus preparations after each treatment were controlled by light and electron microscopy.

4.2.5. Isolation and purification of the INCLUSION BODIES of the nuclear-polyhedrosis virus of Colias electo and the granulosis virus of Heliothis armigera.

A. The dead caterpillars were suspended in water in a glass beaker and cut up into small pieces with sterile scissors. Antibiotics were withheld because bacterial putrefaction was desirable, but an antifungal agent, mycostatin, 25 units/ml

was added. The Colias electo larval suspension was allowed to stand at room temperature for 1-3 weeks. The Heliothis armigera caterpillars were allowed to decompose for 1 week only. The top layer of debris was then removed and the supernatant fluid evacuated by suction. The resultant whitish sediment of inclusion bodies and some debris which settled were resuspended in water and filtered through cheese cloth.

B. Differential centrifugation was carried out at low and high speeds. The optimal high speed was the lowest necessary to collect the majority of the inclusion bodies in a packed pellet and the optimal low speed was the highest possible to sediment non-viral material and leave the majority of the polyhedra in the supernatant. Thus the polyhedral suspension of Colias electo was centrifuged at 2,000 rpm for 1 min followed by 7,500 rpm for 5 min for two cycles. The sample of Heliothis armigera was sedimented at 2,000 rpm for 5 min followed by 12,500 rpm for 10 min for three cycles.

C. The organic solvent used was the fluorocarbon, trichlorotrifluoroethane (Arcton 113, Imperial Chemical Industries). The inclusion bodies were suspended in distilled water and mixed with an equal amount of fluorocarbon. The preparation was then vigorously shaken by hand for about 5 min until a homogeneous emulsion was formed. The separation of the water and fluorocarbon phases was assisted by low speed centrifugation, 2,000 rpm for 30 sec for the preparation of Colias electo and 2,000

rpm for 1 min for the preparation of Heliothis armigera. After a thin layer of insoluble lipids was removed from the surface, the aqueous suspension containing the polyhedra was collected. Inclusion bodies were trapped in the denatured non-viral fragments at the interface and three successive washings with distilled water released them to the aqueous top layer. All the top aqueous layers were collected, centrifuged at the appropriate speed to sediment the inclusion bodies; 7,500 rpm for 5 min for the polyhedra of Colias electo and 12,500 rpm for 10 min for the granulosis capsules of Heliothis armigera. The pellet was resuspended in 5 ml of distilled water and the procedure was repeated twice for the polyhedra of Colias electo and three times for the granulosis capsules of Heliothis armigera.

D. Lyophilization was carried out in an apparatus (Fig. 17) composed of a manifold with openings to which ampoules were attached by rubber tubing. At one end the tube was sealed and at the other end connected to a large flask surrounded by dry ice and attached to a vacuum pump. The pyrex ampoules of 10 or 25 ml capacity, half filled with inclusion body suspension, were connected to the apparatus and were cooled to about -72°C by a mixture of dry ice and acetone poured on the outer walls, until a frozen layer was formed. The vacuum pump was then operated overnight, by which time the suspensions of the inclusion bodies had evaporated to a dry powder. The ampoules were then sealed with an oxygen-acetylene flame and were stored at 4°C.

Freeze-drying was done prior to the ultrasonic

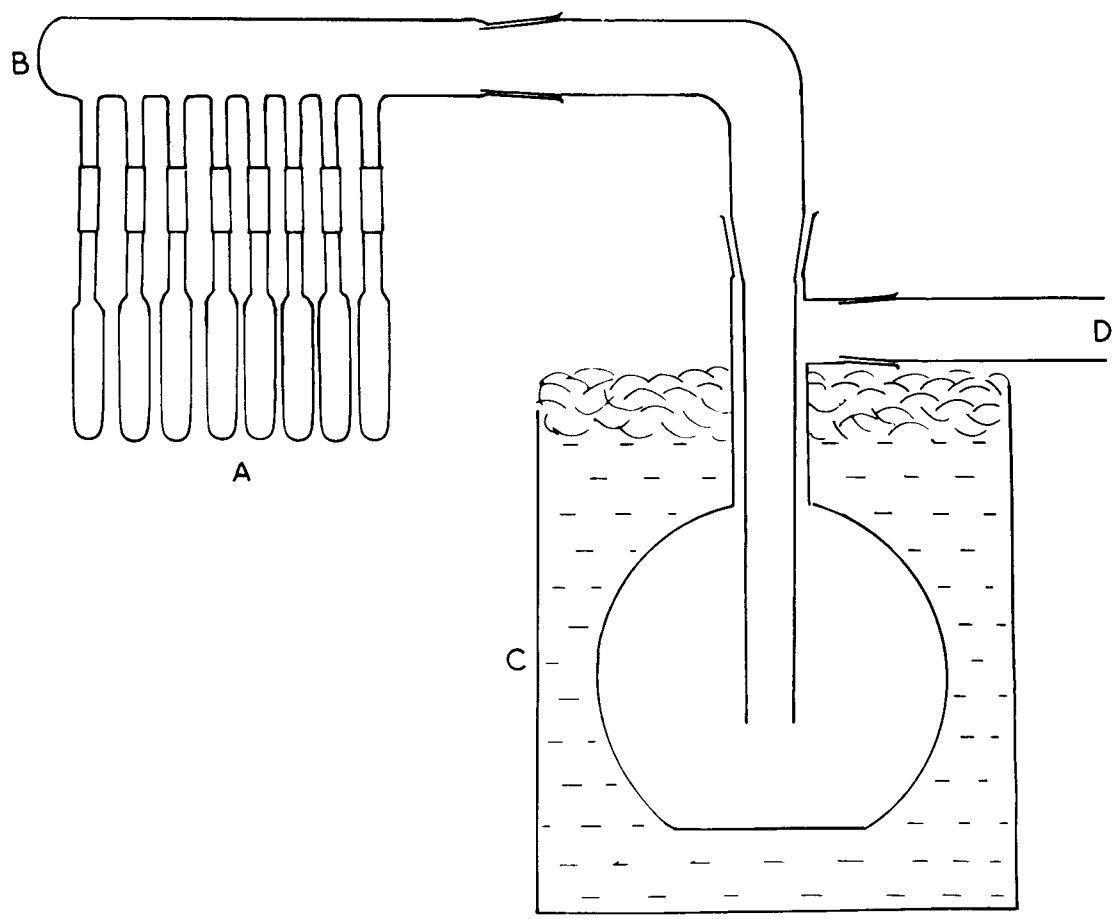
Fig. 17. Freeze-drying apparatus.

A. Ampoules with inclusion body suspensions.

B. Manifold.

C. Dry ice container.

D. Tube to vacuum pump.


B
A
D
C

treatment, so that if aggregates were formed during lyophilization, they could be dispersed by the subsequent sonication. Furthermore, an accurate weighing of the freeze-dried material was possible and this is one of the critical conditions for successful sonication.

E. Sonication of the inclusion bodies was carried out in a Branson Sonifier model S 110. The sonifier had a sonic power supply operating at a frequency of 20 kc/sec. The power output may be set at 8 different levels indicated by the numerals 1 to 8. These ranged between 0-75 watts. A gauge measured the amperage (1-15 amp) and a knob adjusted the intensity. The energy was focussed at the tip of the probe, 12.5 mm diameter. The probe was dipped 1 mm into the suspension just sifficient to break the meniscus. Bubble formation accompanied by a hissing noise was a general indication that cavitation had occurred.

Each sample, weighing 2 g when freeze-dried, was suspended in 10 ml distilled water and was placed in a vial, 5 cm long and 2.5 cm diameter. The vial was surrounded by crushed ice during sonication. The temperature of one representative suspension was measured before and after the treatment and the temperature rise was insignificant being from 20 to 22°C. This was due to the adequate cooling of the container. The optimal sonication conditions for both viruses were found to be at a power level 5 (50 watts) and intensity of 7.5 amp. The duration of treatment varied and 10 min was found to be the optimal period for the polyhedra of Colias electo and 20 min for the granulosis capsules of

Heliothis armigera. Ten minutes was the maximum time for continuous operation owing to the problem of heat generation. The number and integrity of the inclusion bodies was checked by light microscopy. The integrity of the virus particles was examined by electron microscopy after digestion of the sonicated inclusion bodies.

F. Centrifugation in a sucrose density gradient was used for further purification of the polyhedra of Colias electo and the granulosis capsules of Heliothis armigera. This was unsuccessful. A glass centrifuge tube 17.5 cm long and 2.5 cm diameter was used. A gradient was formed of 2-40% sucrose with intervals of 2% between steps of 3 ml. EDTA 0.01 M was added to prevent clumping and sodium azide, Na_2N (0.2%) was added as a preservative. The gradient was formed from the bottom of the tube and was allowed to stabilize for 2 hr. Three ml of the inclusion bodies in distilled water was applied to the top of the gradient. The tube was centrifuged at 1,500 rpm for 50 min.

4.2.6. Purification of the INCLUSION BODIES of the nuclear-polyhedrosis virus of Heliothis zea.

A. Following Estes and Faust (1966) who used a similar preparation, the inclusion bodies were suspended in distilled water and were sonicated at 20 kc/sec at power level 5 (50 watts), at 7.5 amp for 5 min under conditions similar to those described in 4.2.5.E. After ultrasonic treatment, the residual aggregates were sedimented at 2,000

rpm for 2 min, resuspended in 10 ml distilled water and four further cycles of sonication and centrifugation were carried out. All the supernatants containing the free polyhedra were then combined, sedimented at 10,000 rpm for 10 min and resuspended in 10 ml distilled water. Sonication was repeated on this suspension only once as repetitions were found to be harmful to the suspension of singly dispersed polyhedra. The suspension of the free inclusion bodies was once more centrifuged, resuspended in distilled water and freeze-dried for storage. However, the recovery of inclusion bodies was low after lyophilization. It was therefore avoided thereafter and sonication of the commercial preparation was carried out just prior to subsequent treatments.

Several other methods were attempted to destroy the powder matrix of the commercial preparations of the inclusion bodies of Heliothis zea and non-viral material without affecting the polyhedra but all were unsuccessful. They are listed below.

B. Aqueous suspensions of the polyhedra were mixed with equal amounts of either chloroform or fluorocarbon. The chloroform preparation was mixed in a blender for 30 sec while the fluorocarbon preparation was mixed by hand-shaking for 5 min. The subsequent procedure was similar to the one described for Colias electo polyhedra (4.2.5.C.) but performed once only.

C. An attempt was made to separate the polyhedra from contaminating particles present in the suspension by making use of separation in a triple-cavity thin layer rotor (Polson, to be published). The rotor contains three concentric cavities connected by valves, each of which is adjusted to open at a set velocity. The crude suspension of polyhedra, 0.5 g in 36 ml phosphate buffer of 0.15 M and pH 7.0 was introduced into the central cavity and the rotor was centrifuged at 2,500 rpm for 15 min. At this speed the large particles sedimented against the inner wall of the cavity and the first valve remained closed. By accelerating the speed to 3,000 rpm, the valve opened, allowing the supernatant fluid containing the unsedimented smaller particles to pass to the second cavity. The rotor was spun at this velocity for 20 min. The procedure of centrifugation was repeated at 4,000 rpm, at which velocity the second valve opened and the suspension of the smallest particles passed into the last cavity. The rotor was spun at this velocity for 30 min.

D. Digestion of 0.1 g of the commercial preparation was attempted by 0.1% chymotrypsin in 0.15 M phosphate buffer of pH 7.6 at 37°C for 30 and 40 min.

E. Treatment by acid was tried by immersing 1 g powder in HCl 0.001, 0.01 and 0.1 M for 30 min.

4.2.7. Isolation and purification of the VIRUS PARTICLES of Colias electo, Heliothis armigera and Heliothis zea.

A. Isolation and purification of the virus particles from their inclusion bodies may be achieved by dissolving the latter in weak alkali (Bergold, 1964). Sodium carbonate (Na_2CO_3) in 0.05 M NaCl was used for this purpose. The concentration of the alkaline component was varied according to the resistance of the inclusion bodies to digestion with weak alkali. It was found that a solution of Na_2CO_3 when stored in a stoppered bottle showed a lowering of its pH from 10.0 to 9.5 after storage for 1 month and that its ability to digest inclusion bodies was impaired within a few days. It was concluded that by freeing the water into which the Na_2CO_3 is dissolved of carbon dioxide (CO_2) and preventing any CO_2 from dissolving into the alkali, the problem could be solved. This was done by boiling the distilled water before dissolving the salts and by preparing a solution of 10-fold higher molarity through which nitrogen was bubbled for 10 min and which was kept in a bottle stoppered with a tube containing soda lime. The apparatus is shown in Fig. 18. The solution was diluted with boiled distilled water immediately prior to use. The digestion was performed in stoppered tubes.

The molarity of the alkali solution and the duration of exposure to it were determined roughly by applying a drop of the alkali to a drop of a suspension of inclusion bodies on a microscope slide. The preparation was examined

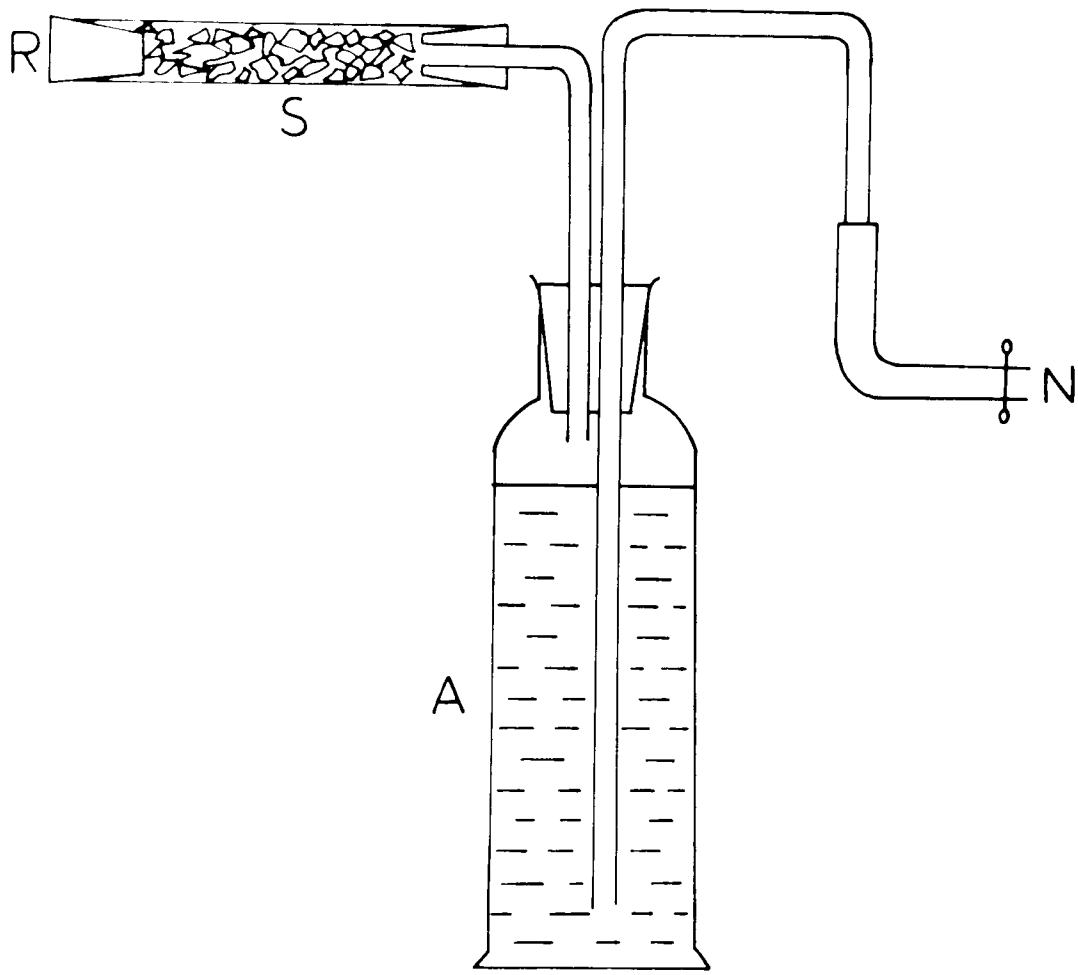
Fig. 18. Container for CO_2-free alkali solution.

A. Alkali solution, Na_2CO_3 + NaCl.

N. Tube to nitrogen cylinder.

S. Soda Lime chamber for CO_2 absorption.

R. Rubber stopper fitted after nitrogen has been bubbled through the alkali solution.


R
S
A
N

microscopically every 15 min over a period of 2 hr under dark field illumination at a magnification X 1000 using an oil immersion objective. For a more accurate determination, the released virus particles and the residual inclusion bodies were examined by electron microscopy after exposure to solutions of different concentration of alkali for different periods.

The nuclear-polyhedra of Colias electo and Heliothis zea were used at a constant concentration of 5 mg of the lyophilized material to 1 ml alkali solution. Higher concentrations of crude viral material were avoided. A solution comprised of 0.02 M Na_2CO_3 + 0.05 M NaCl at pH 10.0 was used as the digesting medium. The polyhedra were exposed to digestion in different cellulose nitrate tubes for 30, 45, 60 and 75 min respectively and the supernatants were combined after centrifugation. Following the collection of the supernatants containing the liberated virus particles, a second term of digestion was carried out on the residual inclusion bodies for an additional 30 min. On completion of each digestion period the volume was adjusted to 10 ml with distilled water and residual inclusion bodies were sedimented, those of Colias electo at 7,500 rpm for 5 min and those of Heliothis zea at 10,000 rpm for 10 min. The resultant pellets were resuspended in distilled water and sedimented again. Thus the residual virus particles were released into the supernatant (Estes and Faust, 1966). The two supernatants obtained after the foregoing centrifugation were combined and centrifuged at 20,000 rpm for 30 min to sediment the virus particles. The

centrifugal force was increased to 25,000 rpm for 60 min to recover empty viral membranes. The pellets of the virus particles were then suspended in distilled water and again centrifuged at the same centrifugal force as before. The resultant pellets were used immediately for observation or for further treatment.

Digestion of the granulosis capsules of Heliothis armigera was done in 0.05 M Na_2CO_3 + 0.05 M NaCl, pH 10.4, using 5 mg of viral material per 1 ml alkali. The digestion was carried out for 90 min. The residual inclusion bodies were sedimented at 12,500 rpm for 10 min and the resultant pellet digested for a further 30 min. After each digestion term, 10 ml distilled water was added and the capsules sedimented once more. The two supernatants were combined and the virus particles were sedimented at 20,000 rpm for 30 min.

Several other procedures were tried to further purify the virus particles, but were found to be unsuitable. The following methods were tested:

B. Resuspension of the nuclear-polyhedrosis virus of Heliothis zea in EDTA buffer of 0.01 M and pH 7.4 was tried. It was thought that by preventing aggregation of the virus particles and the polyhedral protein, a purer preparation would be obtained.

C. Vigorous trituration and resuspension of the sedimented pellets of the nuclear-polyhedrosis virus of

Heliothis zea in distilled water or in phosphate buffer of 0.15 M and pH 7.0 was attempted.

D. Separation of the nuclear-polyhedrosis virus of Heliothis zea was attempted by centrifugation at 30,000 rpm in the SW 39 rotor for 2 hr in a caesium chloride gradient. The gradient was prepared according to the method of Polson and Levitt (1963). Because of uncertainty regarding the density of the virus particles, their suspension in phosphate buffer was introduced on top of the gradient.

4.3. RESULTS

In all procedures directed towards the isolation of virus particles, it is highly desirable to have an objective, accurately reproducible method of assay or quantitation of the particles under study.

Owing to the lack of regularly available susceptible insect hosts and of susceptible cells in artificial culture, the degrees of purification and the numbers of virus particles could most conveniently be assessed by direct observation of the material by light and electron microscopy.

The results of attempts to purify the inclusion bodies and the virus particles are listed in Tables 4.3.1., 4.3.2., 4.3.3. and 4.3.4.

In the next chapter the results of further purification of the inclusion bodies and virus particles by zone electrophoresis in a sugar gradient are presented (Chapter 5).

Table 4.3.1. Isolation and purification of the INCLUSION BODIES.

Treatment	Polyhedra of *Colias electo*	Capsules of *Heliothis armigera*	Polyhedra of *Heliothis zea*
Releasing the inclusion bodies from the insects	4.2.5.A. Decomposition in water for 1-3 weeks followed by filtration through cheese cloth resulted in the removal of coarse tissue fragments.	4.2.5.A. Decomposition in water for 1 week followed by filtration through cheese cloth achieved separation from coarse fragments of debris.	The isolation and first stage of partial purification of this commercial preparation had been done by the manufacturers.
Differential centrifugation	4.2.5.B. Two cycles of differential centrifugation resulted in further partial purification of the polyhedra by removal of many bacteria and fragments of debris.	4.2.5.B. After 3 cycles of differential centrifugation further separation of the capsules from residual debris and bacteria was achieved.	4.2.6.A. Separation of the single and aggregated inclusion bodies from the matrix in which they were distributed by the manufacturers could not be achieved by centrifugation alone. Prior sonication was necessary (see below). Five cycles of sonication each followed by a cycle of differential centrifugation separated the singly dispersed polyhedra from the aggregates.

Table 4.3.1. (Continued)

Water-organic solvent phase system	4.2.5.C. Further purification was achieved by 3 cycles of shaking with fluorocarbon. More debris was removed and some of the polyhedral aggregates were dispersed. A fourth treatment resulted in a loss of polyhedra.	4.2.5.C. Four cycles of treatment with fluorocarbon were effective in further reduction in the number of bacteria and the amount of debris, without damaging the capsules. Some aggregates were dispersed. Loss of capsules was noted after a fifth treatment.	4.2.6.B. Agitation in fluorocarbon or chloroform proved to be too drastic for these polyhedra and failed to disperse the aggregates of polyhedra with residual matrix. The procedure was therefore not used.
Lyophilization	4.2.5.D. Effective for storage at 4°C.	4.2.5.D. Effective for storage at 4°C.	4.2.6.A. Harmful to the integrity of the polyhedra.
Sonication	4.2.5.E. One cycle of 10 min resulted in dispersion of aggregates and disruption of most residual bacteria. A further cycle of 5 min caused visible damage to the polyhedra.	4.2.5.E. A 20 min cycle was effective in dispersing the aggregates of capsules with bacteria. An additional cycle caused damage to the capsules.	4.2.6.A. Five cycles of 5 min each followed by differential centrifugation each time successfully released the polyhedra from the matrix and almost completely dispersed the aggregates.

Table 4.3.2. Unsuccessful methods of purification of the INCLUSION BODIES.

Treatment	Polyhedra of *Colias electo*	Capsules of *Heliothis armigera*	Polyhedra of *Heliothis zea*
Centrifugation in a sucrose gradient	4.2.5.F. No concentration bands were seen and polyhedra were found widely dispersed throughout the gradient column, admixed with non-viral material.	4.2.5.F. No virus bands were observed in the gradient column and capsules were seen by electron microscopy in all fractions taken from the column.	N D
Thin layer centrifugation in a triple-cavity rotor	N D	N D	4.2.6.C. Fractionation was not achieved. Polyhedra and non-viral components were found in all three cavities of the rotor.
Digestion with chymotrypsin	N D	N D	4.2.6.D. In an attempt to digest the powder matrix selectively, chymotrypsin proved to have no effect on the matrix, nor on the polyhedra.
Acid treatment	N D	N D	4.2.6.E. 0.001M and 0.01M HCl had no effect on the polyhedra or the matrix. Both were digested by 0.1 M HCl.

N D = Not done.

Water-organic solvent phase system (4.2.6.B.) and lyophilization (4.2.6.A.) which were not successful for purification of the polyhedra of *Heliothis zea* are discussed in Table 4.3.1.

Table 4.3.3. Isolation and purification of the VIRUS PARTICLES.

Treatment	Virus particles of *Colias electo*	Virus particles of *Heliothis armigera*	Virus particles of *Heliothis zea*
Alkali digestion of the inclusion bodies and release of the virus particles	4.2.7.A. Increasing periods of exposure to alkali resulted in increasing degradation of the virus particles. After 30 min intact virus particles were numerous, but longer periods up to 75 min increased the proportion of empty membranes and free nucleoprotein. By differential centrifugation virus particles could be partially separated from residual polyhedra and from the inclusion body protein fragments and empty membranes. Further purification was achieved by zone electrophoresis (see Chapter 5).	4.2.7.A. Periods of digestion by alkali of less than 2 hr released some of the virus particles. Longer periods of digestion from 2-3 hr effected the release of more virus particles from the capsules but the virus particles showed increasing damage.	4.2.7.A. Controlled periods of alkali digestion resulted in digestion of the polyhedra and release of virus particles, as described for *Colias electo*, but much larger amounts of polyhedral insoluble protein fragments were associated with the released virus particles of *Heliothis zea*.

Table 4.3.4. Unsuccessful methods of purification of the VIRUS PARTICLES.

Treatment	Virus particles of Heliothis zea
Resuspension in EDTA buffer	4.2.7.B. The virus particles appeared to disintegrate.
Vigorous trituration and resuspension of sedimented pellet of virus particles	4.2.7.C. The virus particles were disrupted. This method, however, was used for preparing electron micrographs of the released nucleoprotein fragments.
Separation by centrifugation in a caesium chloride gradient	4.2.7.D. Visible bands could not regularly be produced even after prior purification of the polyhedra by zone electrophoresis (Chapter 5). Electron micrographs of bands which did appear revealed only membranes of virus particles and thin 'virus-like' structures (see 6.3.3.).

4.4. DISCUSSION

The purpose of these experiments was to obtain pure and infective viral elements. While purity was checked by light and electron microscopy, infectivity tests were not possible because a virus-free stock of caterpillars was not available. It is assumed that, as inclusion body and virus membranes protect the infective nucleic acid, the presence of intact virus particles and intact inclusion bodies ensure infectivity. It is known, however, that nucleic acids of insect viruses are infective only when injected in a combination with the polyhedral protein, suggesting a role for the latter in invasion (Smith, 1967). The assumption

that the virus particles are infective is, therefore, based on the work of others, where similar purification procedures were used and the virus particles were still found to be infective.

In the course of this study it was noticed that in each step of purification, the smaller the number of inclusion bodies used, the more effective the procedure. This was also pointed out by Van der Geest (1968) who found it necessary to develop a special purification technique for large quantities of nuclear-polyhedra. In the present study, however, this technique was not necessary as only small amounts of virus were needed for further treatment by zone electrophoresis (Chapter 5) and for electron microscopy (Chapter 6).

When the dead insects decomposed in water, preservatives were withheld in order to allow putrefaction to take place and thus bacterial growth was often excessive. The only way of avoiding this could have been by homogenizing the sick and dead larvae at an early stage. The disadvantage of the homogenizing procedure was that the debris was broken up into small fragments that separation of the virus particles by differential centrifugation was difficult. This was a serious problem in using the nuclear-polyhedra of Heliothis zea which were commercially prepared by homogenization followed by incorporation in a powder matrix. On balance it seemed that the chances of obtaining a purer preparation were increased by allowing the insects to

putrefy and then attempting to eliminate the bacteria.

The persistence of aggregates was a major problem in purification. Contaminating substances like the powder in the commercial preparation of Heliothis zea simply held the particles together in a matrix. Alternatively, proteinaceous contaminants adhered to the surface of the virus particles or inclusion bodies through coulomb forces. It appeared that a cycle existed whereby contaminants caused aggregation or viral elements and in the process of their clumping a further trapping of contaminants occurred. On the other hand, when the preparation was too highly purified there was also a tendency to undergo spontaneous aggregation (Gregory et al., 1969). In this case, the reduction of surface charges by chelating agents and salt solutions could be expected to reduce or inhibit the aggregation. A chelating agent, EDTA, was successfully used to prevent aggregation of partially purified inclusion bodies during zone electrophoresis (5.2.2.C.), but as virus rods disintegrated in the presence of EDTA (Table 4.3.4.) it could not be used for dispersing aggregates of virus particles (4.2.7.B.). Salt solutions cannot be used to prevent aggregation because the inclusion bodies are known to be highly sensitive to salts (Bergold, 1953). Even in cases where chelating agents and salts could effectively be used, a prior dispersion was necessary if reaggregation was to be prevented. This was achieved by shaking with fluorocarbon, and to a greater extent by sonication.

Shaking the inclusion bodies with fluorocarbon achieved only partial purification, a fact which was also observed by Entwistle and Robertson (1968). However, Bergold (1959) achieved a highly purified preparation in this manner. In fact the method was more efficient in the initial purification of crude material, when the particles were protected from surface denaturation by contaminating material (Philipson, 1967). For this reason the number of repeated treatments had to be limited. This phenomenon was demonstrated in this study (Table 4.3.1.) when the capsules of Heliothis armigera virus, which were heavily contaminated with bacteria, withstood four repeated treatments, the Colias electo inclusion bodies withstood three and the polyhedra of Heliothis zea which had been purified beforehand by the suppliers, were inactivated in the first treatment. When the organic solvent was used after sonication the polyhedra were even more sensitive to fluorocarbon.

Sonication proved to be harmful to free virus particles and this has been noted by Bergold (1953, 1964). However, inclusion bodies exposed to a suitable intensity of vibrations for a limited period were not damaged. Once the appropriate intensity and duration were determined, the other factors were kept as constant as possible, e.g. the size and concentration of the sample, the related viscosity, frequency of vibration, diameter of the probe, its depth of insertion and the temperature during sonication. Heating, which results directly from the sonic vibration, was

controlled by cooling so that the rise in temperature after 10 min continuous exposure was only from 20°C to 22°C. Without cooling, Whitcomb et al. (1968) reported a rise of temperature of 13°C after 1 min. In general, the longer the treatment the more inactivation occurred, as was demonstrated in this study with Colias electo and Heliothis armigera inclusion bodies (Table 4.3.1.). The type of cells treated also determines the degree of inactivation. The granulosis capsules were more resistant to sonication than the Colias electo polyhedra and the singly dispersed polyhedra of Heliothis zea. Since the capsules also required a higher molarity of alkali and a longer time for digestion it is likely that they are more rigid than the polyhedra. The polyhedra of Heliothis zea, when aggregated in the powder matrix, were more resistant than the polyhedra of Colias electo and the capsules of Heliothis armigera, but this may be due to the powder matrix having a protective effect on the particles. Most vegetative bacteria seemed to be destroyed after 5-10 min of ultrasonic treatment. This is in agreement with Bucher (1963) and Faust and Estes (1966). Destruction of bacterial spores, however, requires a much longer exposure to ultrasound and may account for the persistence of some bacteria.

Freeze-drying was found to be an efficient way of storing the inclusion bodies of Colias electo and Heliothis armigera. The freeze-dried nuclear-polyhedra of Heliothis zea were apparently damaged by this procedure and could not be detected after resuspension in water. Since the

Heliothis zea polyhedra had already been through one cycle of lyophilization during the course of preparation, the repeated treatment could have caused their disintegration, in accordance with the view of Bergold (1953).

Attempts to purify the inclusion bodies by centrifugation in a sucrose gradient (4.2.5.F.) and in the triple-cavity rotor (4.2.6.C.) were not successful (Table 4.3.2.). Inclusion bodies were not uniform in size and density and were thus distributed throughout the sample along with bacteria and other impurities which were not sufficiently different in their mass to be separated by these means.

While digesting the preparation of the nuclear-polyhedra of Heliothis zea with a proteolytic enzyme (4.2.6.D.) and strong acid (4.2.6.E.), their resistance to the first and sensitivity to the latter were demonstrated (Table 4.3.2.). This is in agreement with the findings of Bergold (1953, 1964). However, these methods proved unsatisfactory for purification.

For digestion of the inclusion bodies only fresh solutions of alkali kept free of CO_2 were effective (4.2.7.A.). When exposed to CO_2, the drop in pH was due to the reaction $Na_2CO_3 + H_2O + CO_2 \rightleftharpoons 2\ NaHCO_3$. To avoid this reaction, soda lime ($NaOH + Ca(OH)_2 + CaCO_3$) was used to absorb CO_2 by conversion of $Ca(OH)_2$ into $CaCO_3$. Hughes (1950) also noticed the higher efficiency of fresh over old

solutions of alkali and Bergold (1953) recommended digestion in containers tightly closed to avoid exposure to CO_2.

In the digestion procedure, the sensitivity of the two nuclear-polyhedrosis viruses to alkali was very similar. While Estes and Faust (1966) digested the nuclear-polyhedra of Heliothis zea by 0.02 M carbonate for 1 hr, Shapiro and Ignoffo (1969) used 0.01 M for 3 hr for the same virus. These results point out the inverse relationship between the concentration of the alkali and the duration of exposure to it, although the respective combinations may suit one virus better than another. A few workers reported that further degradation of the virus particles occurred by increased alkali concentration or duration of exposure (Bird, 1959; Harrap and Juniper, 1966; Ponsen et al., 1964, 1965). In the present study this was observed to only a limited extent. After digestion for 30 min the number of intact virus particles was greater than in the more prolonged digested samples. After 75 min of exposure the situation was reversed but all stages of degraded virus particles, though in different ratios, were found in all the preparations. When digested on the grid for electron microscopy, Bergold (1964) noticed the same phenomenon. For this reason, instead of using the supernatant of a single sample, the best yield of all stages of degradation was achieved by combining the supernatants of several samples of inclusion bodies exposed to alkali for different periods (Table 4.3.3.).

Shapiro and Ignoffo (1969) reported a loss of 99.99%

infectivity after 3 hr exposure to 0.01 M Na_2CO_3. Accordingly, in each sample digestion was stopped before all the polyhedra were digested, so that the released virions could be removed from the alkali by centrifugation before being damaged. After this centrifugation a further digestion was carried out on the residual pellets (Table 4.3.3.). The combination of a few solutions exposed to alkali for different periods and to the two-term digestion, each of a shorter time, does not appear to have been reported in the literature. The yield of virus particles seemed to be higher using this method.

The granulosis capsules needed a considerably higher concentration of alkali and a longer period of digestion than the nuclear-polyhedra. The use of 0.05 M Na_2CO_3 up to 3 hr was also reported for other granulosis viruses (Steinhaus and Thompson, 1949; Sidor and Krstić, 1969). The fact that very few intact virus particles were present in the granulosis preparation was due to the destructive effect of the high pH to which they were exposed during digestion. Most of these intact particles were within their outer membrane and accordingly less susceptible to alkali, a fact which has also been recorded by Bergold (1953).

A number of other purification techniques were applied to the suspensions of virus particles but were found to be unsuitable (Table 4.3.4.). Although EDTA was used to prevent aggregation of the inclusion bodies, it caused disintegration of virus particles. Centrifugal force followed by vigorous trituration of the sedimented pellet

resulted in disruption of virus particles, a phenomenon observed by Polson and Stannard (1969) with suspensions of influenza virus and pantropic Rift Valley fever virus. Centrifugation in a caesium chloride gradient was unsuccessful because of the damaging effect of the medium on the virus particles of Heliothis zea after long periods of exposure. In retrospect it may have been important to repeat this procedure using the method of Polson and Levitt (1963) in which the time of centrifugation is greatly reduced by the introduction of the virus sample into the gradient close to the isopycnic density of the virus particle. This method has not been attempted again as density gradient zone electrophoresis was found satisfactory for further purification of the virus particles.

CHAPTER 5

DENSITY GRADIENT ZONE ELECTROPHORESIS

5.1. INTRODUCTION

Viruses which have an outer protein coat exhibit a net negative charge at pH values above their isoelectric point and a net positive charge below this point. In an electric field they migrate either towards the cathode or anode, depending on the net charge on the surface of the particles. This movement is known as electrophoresis and the distance travelled is a physical characteristic under constant conditions of each protein or viral component and may be used for characterization, separation and purification.

Since electrophoresis in a uniform or solid medium had proved unsatisfactory in many instances, Brakke (1953, 1955) introduced the sugar density gradient zone electrophoresis in buffer. The main purpose of the density gradient is to prevent mixing by thermal convection, electro-osmotic flow or by mechanical disturbances of fluid in the electrophoresis column. Other advantages are the ability to use small amounts of material which may be detected optically, its relatively quick migration, lack of adsorption to the medium and simplicity of sampling.

According to Brakke (1955), the strength of the electric field increases with the increase in viscosity in

the column. The tendency of the increased viscosity to slow down the rate of migration is therefore balanced by the effect of the increased field strength in speeding it up. As a result, a constant migration rate is achieved which depends mainly on the overall net field strength and it is almost independent of the sucrose concentration and the field strength gradient.

The technique of density gradient electrophoresis is not suitable for measuring absolute electrophoretic mobility values. Relative electrophoretic mobilities of different substances can, however, be determined under standard conditions. Van Regenmortel (1968) suggested relating the mobility of the substances under test to the mobility of phenol red. Unlike the absolute migration value of the virus, this value termed R_{ϕ} is not dependent on standardization of conditions such as the concentration and potential gradient, the temperature, the duration of the experiment and the width of the column. The ionic strength and pH value of the buffer used for diluting the sugar, however, should be kept constant.

The electrophoresis apparatus, originally designed for use with solid media, was successfully used for a liquid medium by Svensson and Valmet (1955). Polson and Cramer (1958) simplified and improved the apparatus described in detail by Polson and Russell (1967).

Density gradient zone electrophoresis has been used in the study of animal and plant viruses for various purposes. Polson and Deeks (1962) and Van Regenmortel (1968) used it

for classification and identification of both enteroviruses and plant viruses, respectively. Electrophoresis was also used to separate viral components (Cramer and Stewart, 1960) and a mixture of viruses (Van Regenmortel and Fowle, 1962; Van Regenmortel et al., 1964) or to purify animal virus (Polson and Cramer, 1958; Polson and Deeks, 1962) and plant virus (Van Regenmortel, 1960, 1961, 1964a, b) from host contaminants. Alternatively, electrophoresis can indicate homogeniety and purity of virus preparations (Cramer, 1959; Van Regenmortel et al., 1964). The only insect virus which has been purified by zone electrophoresis is a nonoccluded virus of the pine tree emperor moth Nudaurelia cytherea capensis (Tripconey, 1970).

The techniques of density gradient zone electrophoresis was used to further purify the inclusion bodies and their viruses which are the subject of this study and to separate the virus particles within the outer membrane from those within the inner membrane alone. The three viruses were also studied in order to determine if they could be distinguished by their relative electrophoretic mobilities, i.e. $R_{\emptyset}$ values.

5.2. MATERIALS AND METHODS

5.2.1. The apparatus.

The zone electrophoresis apparatus, shown in Fig. 19, is similar to the one described by Polson and Cramer (1958). It consists of a U-shaped tube, electrode vessels and a capillary sampling tube.

The left arm of the tube (G) is the electrophoresis column having an internal diameter of 2 cm. The right arm is equipped with a stopcock (J).

A rubber tube is fitted to the bottom of the capillary (L) and provided with a clamp and a terminal Luer-Lok fitting. This is used for the insertion of the gradient and the virus into the column and for sampling at the end of the experiment.

5.2.2. Buffers and solutions.

A. Borate buffer (Polson and Russell, 1967).

(a) Borate stock solution.

H_3BO_3	12.4 g
NaOH	4.0 g
Distilled water to	1 litre

(b) 0.1 N HCl.

HCl (density 1.186 g/ml)	9.1 ml
Distilled water to	1 litre

(c) Normal saline.

NaCl	8.5 g
Distilled water to	1 litre

Fig. 19. Zone electrophoresis apparatus connected to a gradient-forming device.

A. 40% sucrose flask.

B. Borate buffer flask.

C. Connecting rubber tube with capillary and clamp.

D. Magnetic stirrer.

E and F. Electrode vessels.

G. Electrophoresis column.

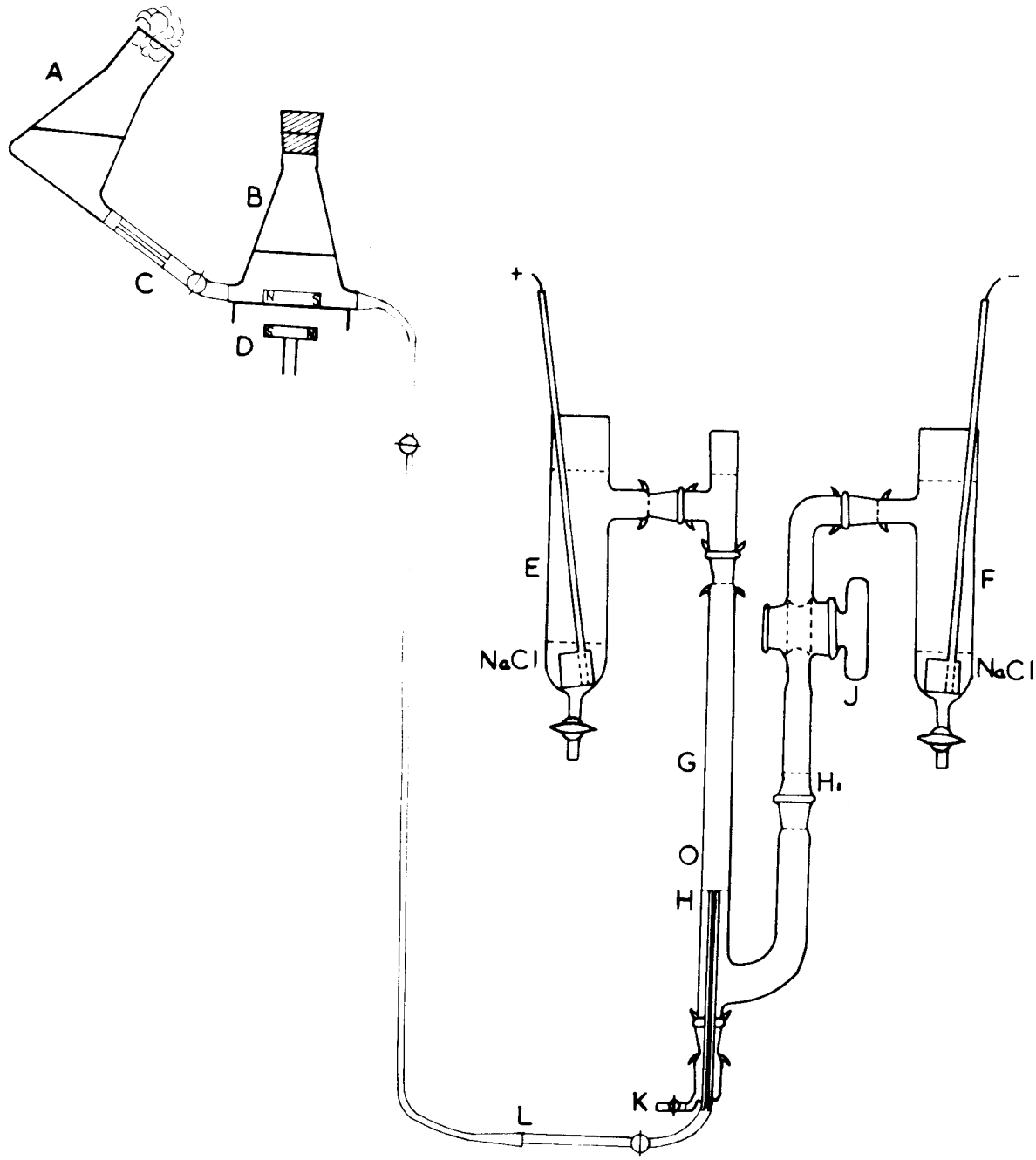
H and H_1. Levels of 40% sucrose.

J. Stopcock.

K. Site of initial filling of the column.

L. End of capillary sampling tube, connected to rubber capillary tube of the gradient-forming device.

O. Origin of the sample.


A
B
C
D
N
S
S
N
+
−
E
F
NaCl
NaCl
J
G
H₁
O
H
K
L

The buffer was made up by mixing:

350 ml of solution (a)

150 ml of solution (b)

500 ml of solution (c)

1000 ml Distilled water

The buffer had a pH of 8.6 and ionic strength of about 0.1.

The final concentration of the buffer was:

H_3BO_3	0.0350 M
NaOH	0.0175 M
HCl	0.0075 M
NaCl	0.0730 M

B. A 40% sucrose solution was prepared by dissolving 160 g analytical sucrose in 200 ml of double strength borate buffer having an ionic strength of about 0.1 which is similar to that of the borate buffer. Due to borate ion-sucrose complex formation, the pH value dropped and was consequently adjusted to a value of 8.6 by the addition of 1 N NaOH.

C. EDTA solution (Ethylenediaminotetra-acetic acid tetrasodium salt, Eastman Organic Chemicals) was used in the electrophoresis experiments of the inclusion bodies. This was done to prevent the formation of aggregates. By dissolving EDTA in double strength buffer at a molarity of 0.01, a final concentration of 0.005 M was prepared in the borate buffer and the 40% sucrose solution. No change of pH was recorded.

All the solutions (A, B, C) were boiled and cooled before use.

D. To ensure that pH 8.6 of the borate buffer had no damaging effect on the inclusion bodies and the virus particles, phosphate buffer of 0.06 M and pH 7.1 was used with representative samples. The results were found similar to those obtained with the borate and for convenience the borate buffer was used.

5.2.3. Initial filling of the column.

The electrophoresis apparatus was thoroughly cleaned with boiling water. The borate buffer was introduced through K and was allowed to fill both arms of the apparatus. The capillary was also filled with buffer by opening the clamp on L for a short time. After all air bubbles had been removed, the 40% sucrose solution was carefully run in through K. When it reached a level of approximately 10 cm above the top of the capillary, the clamp on K was closed and vessel E was filled with buffer. This raised the level of the sucrose solution in the right arm to position H_1. The stopcock (J) was then closed and the sucrose solution allowed to escape through the capillary (L) until the sucrose-buffer interface was defined by a sharp line at level H.

5.2.4. The gradient.

The gradient-forming device shown in Fig. 19 was modified from Polson and Russell (1967). It consists of two flasks, a connecting tube and a long rubber capillary

outlet tube.

Flask A was in a slightly higher position than Flask B and was tilted to allow most of the liquid to flow into Flask B. Flask A was filled with 150 ml 40% sucrose solution in buffer and was loosely plugged with cotton wool.

Flask B was fitted with a sterilized magnet and placed on a magnetic stirrer. Borate buffer, 160 ml, was then added and the flask was tightly closed with a rubber stopper.

The connecting rubber tube (C) was provided with a clamp which was kept closed until gradient formation started. A capillary was inserted to regulate the sucrose flow from flask A to B to the same rate as that of the mixture entering the column.

Immediately after opening the clamp on C and operating the magnetic stirrer, the thin outlet tube was connected by a Luer-Lok fitting to the capillary (L), from which air had been previously removed. The clamp on L was opened and the gradient allowed to flow slowly into the electrophoresis column. As the gradient liquid became more viscous, the tension of the clamp was adjusted to maintain the same rate of flow. The gradient formed in this way is logarithmic, although in the range used it is approximately linear (Polson and Deeks, 1962). The only steep decline in the gradient is near level H and this increases the stability in the region most disturbed by the inflow (Polson and Russell, 1967). Approximately 45 min was found necessary for the formation of a satisfactory gradient. When nearly all the sucrose solution had flowed from flask A to B, the clamp on L was closed and the gradient-forming device disconnected from the apparatus.

5.2.5. The inclusion body and virus suspensions.

Both inclusion bodies and virus particles were subjected to electrophoresis. The inclusion bodies of Colias electo, Heliothis zea and Heliothis armigera were obtained in each case from 10 mg of the freeze-dried material which had been partially purified as described in 4.2.5. and 4.2.6. The virus particle preparations of the three strains were obtained from the digestion of 15 mg of the respective freeze-dried material.

After centrifugation the sedimented pellets of the inclusion bodies were resuspended in 3 ml of 37% sucrose in borate buffer and the virus pellets in 2 ml of 37% sucrose solution. A few grains of the reference substance, phenol red, were added to each sample to give it a red colour. A drop of the mixture was added to 5 ml of 40% sucrose and to 5 ml of a sample taken from the column through the capillary (L) (approximately 35% sucrose). The density of the virus sample was satisfactory if it floated on the 40% sucrose solution and sank in the 35% sucrose solution. This meant that when inserted into the electrophoresis column, the virus sample would rest on the 40% sucrose 'shelf' and not rise through the gradient.

Two ml of each inclusion body sample and 1.5 ml of each virus preparation were applied to the column in different experiments using a 5 ml hypodermic syringe which was screwed onto the Luer-Lok fitting on L. Air bubbles were removed and the sample was inserted slowly and carefully by the syringe. When all the sample had been introduced, the clamp was closed. Vessel F was then filled with buffer to

a level which would force the sample up to level O on the opening of stopcock J. At this point the stopcock was closed and the residual sample in the capillary was run out. Thereafter a period of 3 hr at 4°C was allowed for the gradient to form throughout the sample layer. This was done so that convection would not occur in the initially homogeneous sample layer. The width of the sample band was 1-2 cm.

5.2.6. Electrophoresis.

Reversible silver/silver chloride electrodes were used alternately in the two electrode vessels (E and F). The Ag electrode placed in vessel E formed the positive pole and the AgCl electrode placed in vessel F formed the negative one. Saturated NaCl in water was introduced from below into the electrode vessels in sufficient volume to cover the electrodes. Stopcock J was opened and the height of buffer in the two vessels was adjusted so that the sample stayed at position O. This position was marked on the column.

Electrophoresis was carried out at 4°C. A current of 20 ma at a potential gradient of 3.5 v/cm was passed through the column for a period of 16-26 hr, the electrophoresis column being cooled by a fan.

On completion of the electrophoresis the current was switched off, the stopcock (J) closed and the electrodes removed.

5.2.7. Observations and sampling.

The electrophoresis column (G) was examined in a dark room by illumination with an argon-mercury fluorescent lamp

(Polson and Russell, 1967). The U-shaped lamp had the same length as the column and was attached to a dark plate with a slit between the two arms of the lamp. It was placed as close as possible to the column and parallel to it so that the bands could be examined by light scattering. Photographic records were made and the positions of the phenol red and of the bands of opalescence were recorded.

A centimetre tape was attached to the column and 1 cm long fluid samples were collected from the top of the column using a finely drawn pasteur pipette. The rest of the material was sampled through the capillary at the bottom (L) at 1 cm intervals along the column. Sampling from both ends reduced 'tailing' of the material passing through the capillary. Each sample was collected in a clean cellulose nitrate centrifuge tube and was diluted to 10 ml with distilled water. The sample was then centrifuged at the velocity known to sediment the inclusion bodies or virus particles from the viscous sucrose solution. The resultant pellets were washed once by centrifugation in distilled water and were examined by electron microscopy.

5.3. RESULTS

The records of the results of attempts at further separation of the partially purified suspensions of inclusion bodies or virus particles from non-viral material by electrophoresis are shown diagrammatically in Figs. 20 and 30.

5.3.1. Electrophoresis of the INCLUSION BODIES.

A. The nuclear-polyhedra of Colias electo formed two opalescent bands (Fig. 20A, I and II). The lower band (I) scattered more light than the upper band (II).

Electron microscopy revealed the majority of the free polyhedra of all sizes at the top of band II but some free polyhedra were seen in all fractions taken from bands I and II. Their number decreased towards the bottom of the column whereas the number of their aggregates increased.

There was a clear difference between the fastest migrating free polyhedra which were apparently completely devoid of membrane and those which lagged behind. Most of the former were more translucent so that the virus rods could be easily seen, while the majority of the latter polyhedra were more opaque.

The distance migrated by the fastest moving polyhedra in a given time was reproducible in repeated experiments and therefore the upper boundary was taken for the estimation of the electrophoretic index $R_{\emptyset}$. The width of bands I and II, however, varied slightly from one experiment to the next.

Electron micrographs of representative samples of the starting material (Fig. 21), the partially purified preparation of Colias electo before electrophoresis (Fig. 22) and a fraction taken from the upper boundary of band II (Fig. 23) show the degree of purification and the dispersion of aggregates achieved.

B. The nuclear-polyhedra of Heliothis zea were found by electron microscopy to be mainly distributed throughout

band II (Fig. 20B). In the lower fractions of this band more aggregates and fewer free polyhedra were found. The number of translucent polyhedra was greater in the upper than the lower fractions of the band. Band II varied slightly in width in repeated experiments, however the upper boundary produced by the fastest migrating polyhedra was remarkably constant in relation to phenol red and was used for the calculation of the $R_{\emptyset}$ value as given for Colias electo polyhedra.

Few free polyhedra were found in band I, but this band included much of the powder matrix of the commercial preparation and of polyhedra aggregated with it.

Electron micrographs of the commercial preparation (Fig. 24) and the partially purified preparation of Heliothis zea (Fig. 25) show polyhedra and polyhedral aggregates before electrophoresis. The dispersion of the polyhedra in the more purified suspension taken from the fraction at the upper boundary of band II is shown in Fig. 26.

C. The samples of granulosis capsules of Heliothis armigera were heavily contaminated by bacteria.

Most of the bacteria and a few of the capsules were found in band I; three different types of bacteria gave different opalescent bands (I_1, I_2 and I_3 in Fig. 20C).

Free capsules were seen only in band II associated with very few bacteria. The relative position of band II to phenol red was reproducible and although the midpoint of this band should have been taken to represent the average distance of migration of the capsules, the distance measured from the origin to the upper boundary of band II

was taken for the calculation of the $R_{\emptyset}$ value for purposes of comparison with the values for the migration of the polyhedra in the other two species.

Electron micrographs of the original extract (Fig. 27) and of the suspension of granulosis capsules of Heliothis armigera used for electrophoresis (Fig. 28) show gross contamination with bacteria and tissue fragments. In contrast, the photographic record of the fraction taken from the uppermost boundary of band II in the electrophoresis column shows a remarkable degree of purification (Fig. 29).

Fig. 20. Diagrammatic representation of the migration of the INCLUSION BODIES in the electrophoresis column.

A. The nuclear-polyhedra of Colias electo.

B. The nuclear-polyhedra of Heliothis zea.

C. The granulosis capsules of Heliothis armigera.

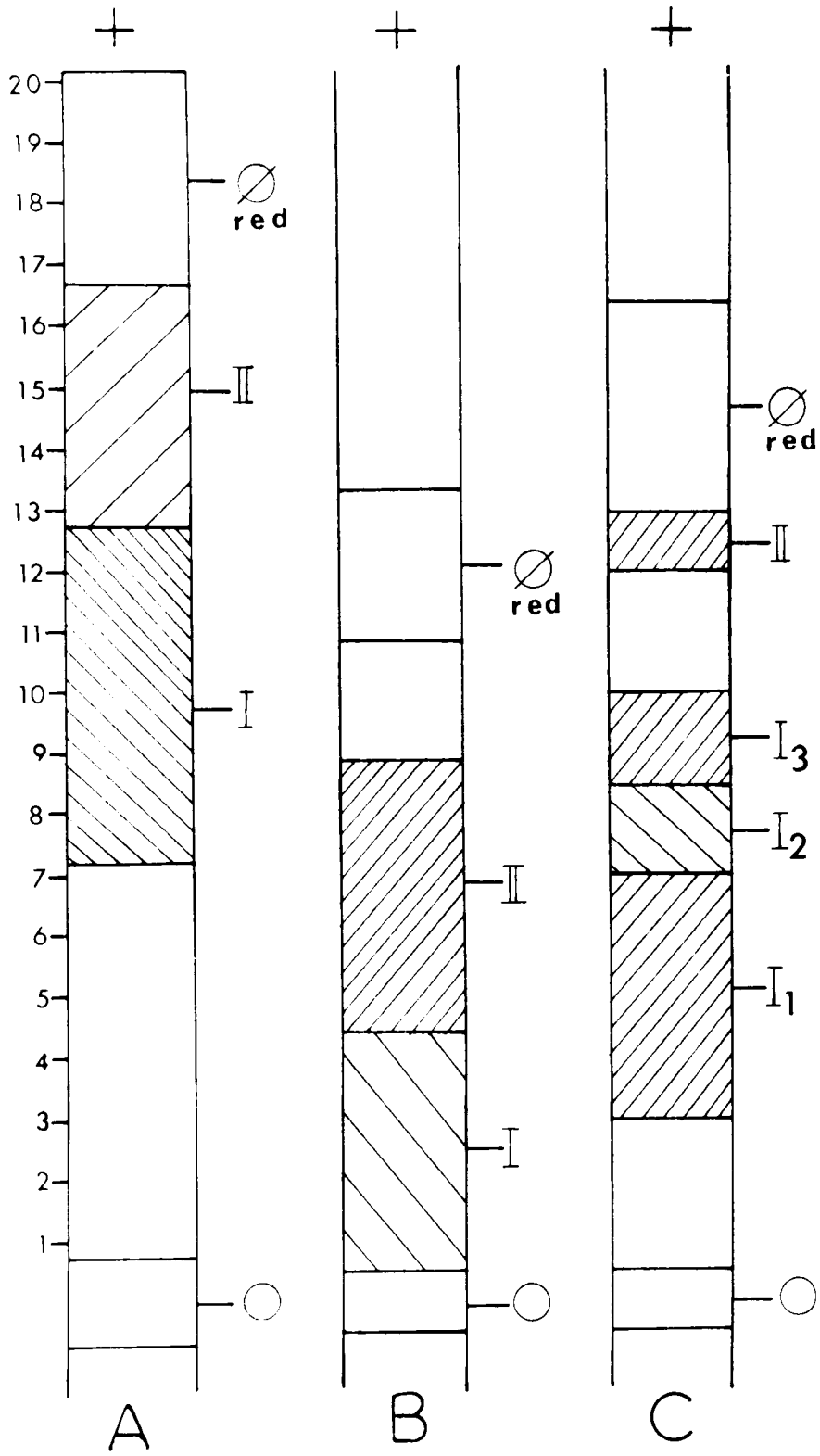
O. Origin.

Ø red. Phenol red.

I. Predominantly aggregates of inclusion bodies (see text).

II. Predominantly free inclusion bodies (see text).

I_1, I_2, I_3. Granulosis capsules aggregated with three different types of bacteria.

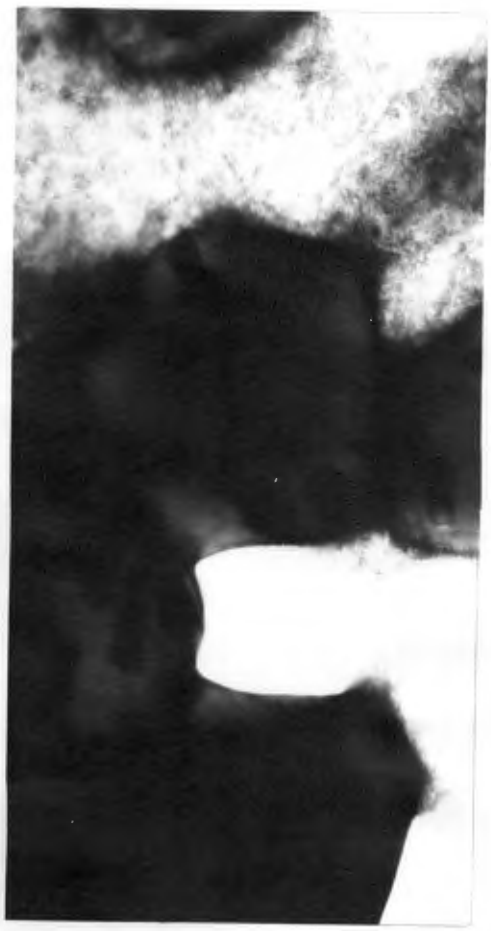

+
+
+
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
∅ red
II
I
∅ red
II
I
∅ red
II
I3
I2
I1
A
B
C

Figs. 21-23. Nuclear-polyhedra of Colias electo. Negatively stained.

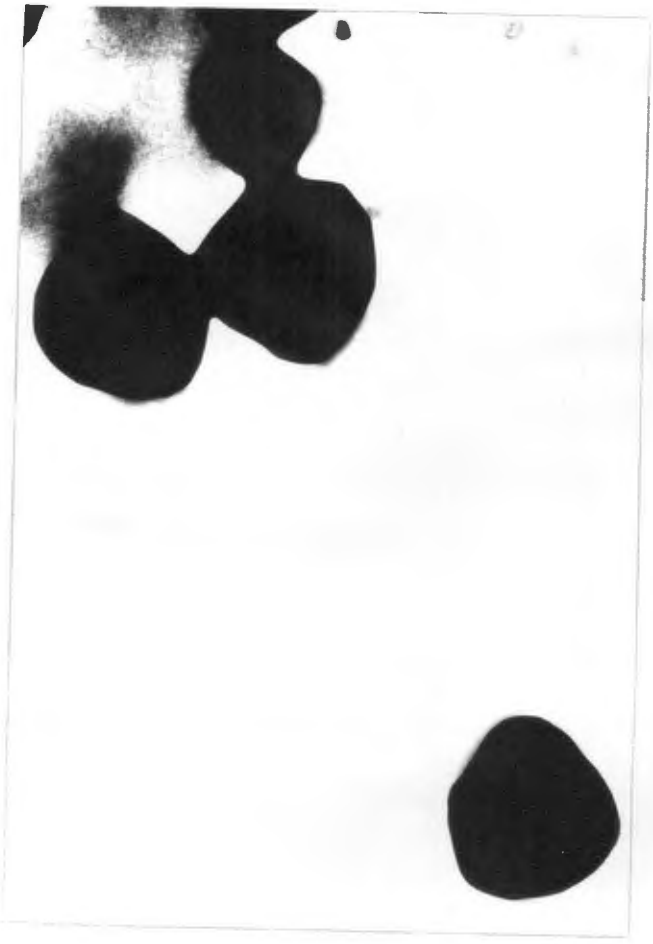
Fig. 21. Polyhedra after release from the insects, before purification. Magnification X 20,000.

Fig. 22. Polyhedra after purification by differential centrifugation, water-organic solvent phase system and sonication. Before density gradient zone electrophoresis. Note the partial dispersion of aggregates and removal of non-viral material. Magnification X 10,000.

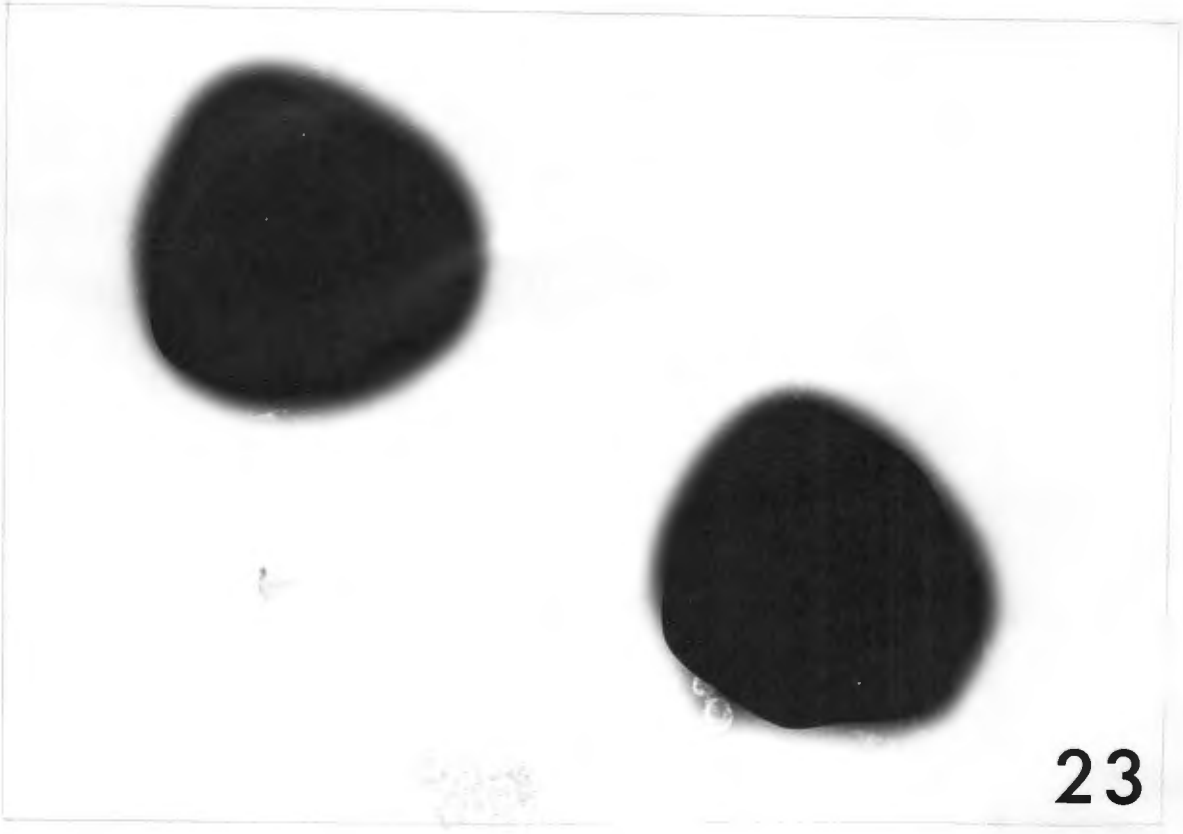
Fig. 23. A sample of polyhedra recovered from the top of band II (fig. 20A) after density gradient zone electrophoresis. Note the elimination of aggregates and further removal of non-viral material. Magnification X 20,000.



21



22



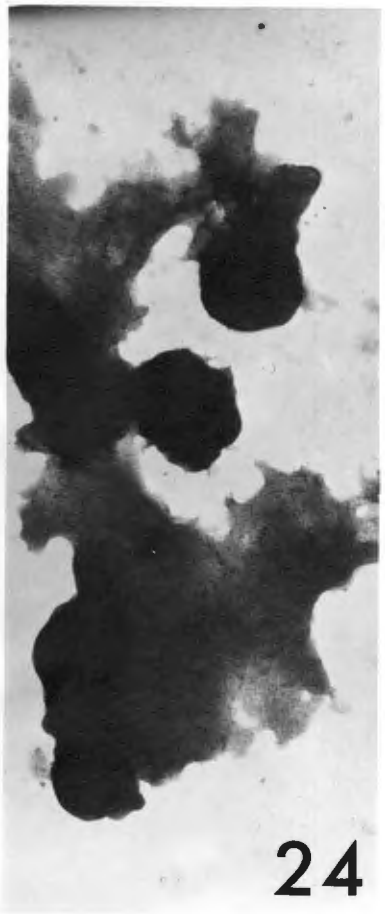
23

Figs. 24-26. Nuclear-polyhedra of *Heliothis zea*. Negatively stained. Magnification X 20,000.

Fig. 24. The commercial preparation of polyhedra before purification.

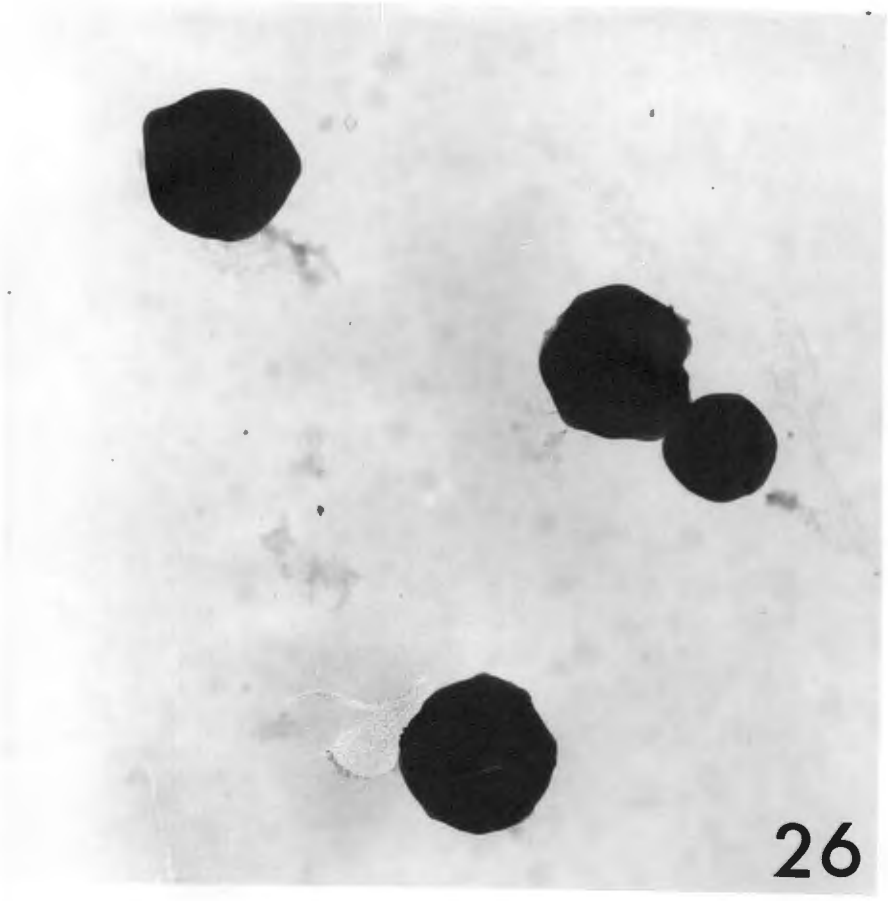
Fig. 25. Polyhedra after purification by sonication and differential centrifugation. Before density gradient zone electrophoresis. Note the dispersion of aggregates and partial removal of non-viral material.

Fig. 26. A sample of polyhedra recovered from the top of band II (Fig. 20B) after density gradient zone electrophoresis. Note the partial elimination of non-viral material.



24

25



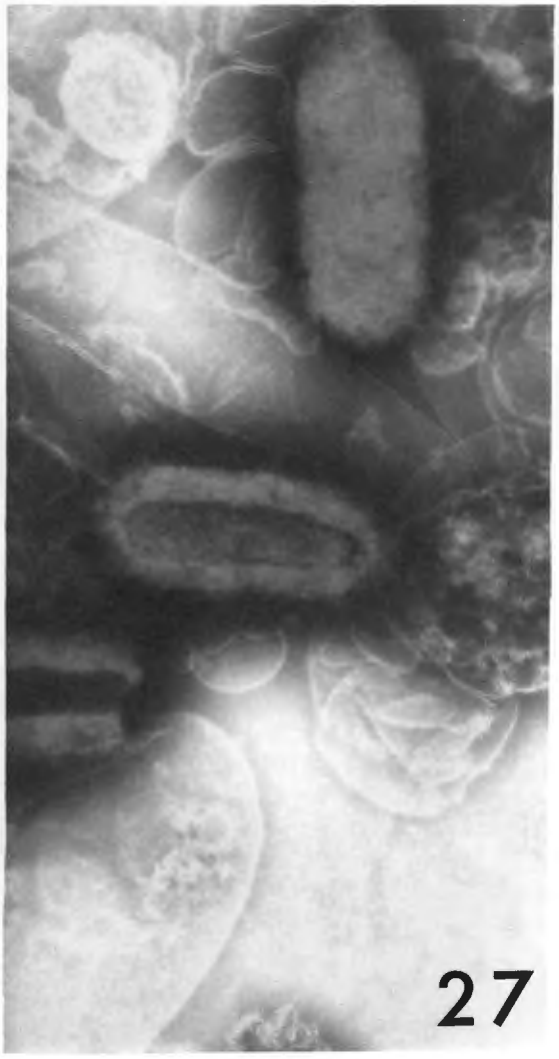
26

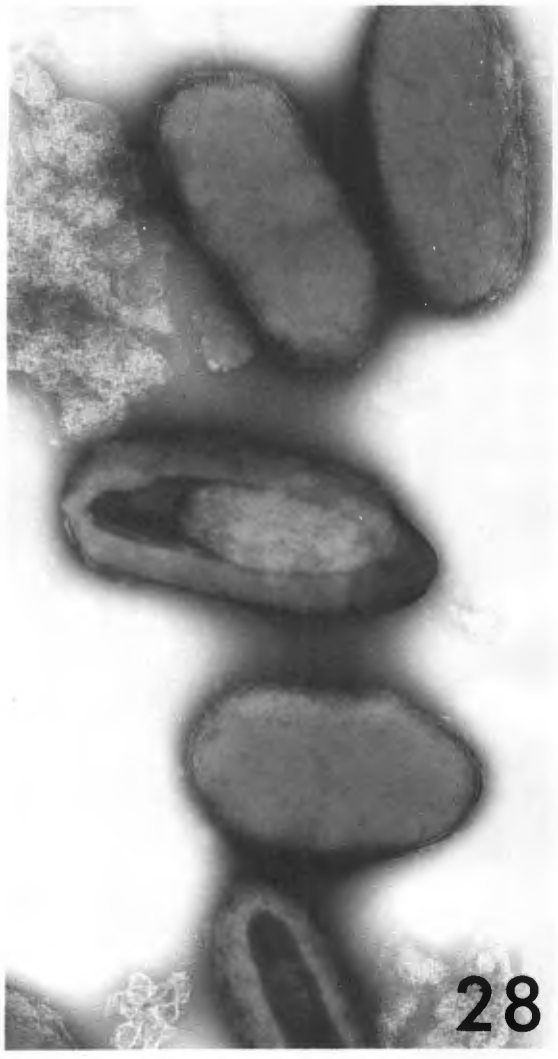
Figs. 27-29. Granulosis capsules of Heliothis armigera. Negatively stained. Magnification X 80,000.

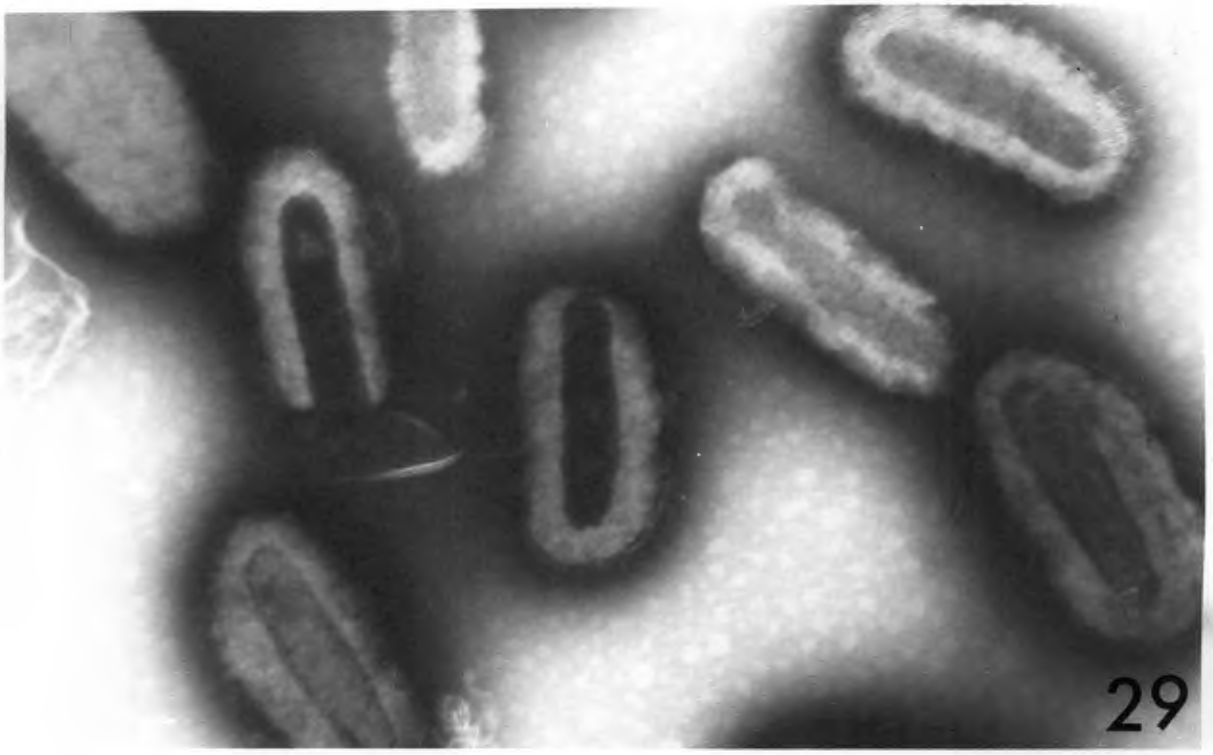
Fig. 27. Capsules after release from the insects. Before purification.

Fig. 28. Capsules after purification by differential centrifugation, water-organic solvent phase system and sonication. Before density gradient zone electrophoresis. Note the partial dispersion of aggregates and removal of non-viral material.

Fig. 29. A sample of capsules recovered from the top of band II (Fig. 20C) after density gradient zone electrophoresis. Note the elimination of aggregates and non-viral material.


27


28


29

5.3.2. Electrophoresis of the VIRUS PARTICLES.

A. The nuclear-polyhedrosis virus particles of Colias electo formed two bands in the electrophoresis column (Fig. 30A). Electron microscopy revealed virus particles within the outer membrane mainly in the upper band II and virus particles within the inner membrane in the lower band I. Empty membranes were found distributed throughout both bands I and II.

In repeated experiments, although two separate bands were always formed, they varied in width and the distance of migration in relation to phenol red. Separation of the two types of virus particles was not complete owing to the varying amounts of the outer membrane remaining attached to the individual particles. The upper boundary of each band was taken to represent the distance of migration of the virus particles with intact outer membranes (band II) and virus particles with only some fragments of outer membrane or no outer membrane at all (band I).

The electron micrographs show representative samples of the unpurified material before electrophoresis (Fig. 31) and virus particles within the outer membrane recovered from band II after electrophoresis (Fig. 32). In Fig. 33 virus particles are seen within the inner membrane and with varying amounts of attached outer membrane recovered from band I after electrophoresis.

B. The nuclear-polyhedrosis virus particles of Heliothis zea also formed two bands in the electrophoresis column but in this instance they were separated from one

another by a less opalescent zone (band III in Fig. 30B).

The two upper boundaries of bands II and I correspond with the purest preparations of intact virus particles within the outer membrane and virus particles within the inner membrane respectively and were therefore taken as the distances of migration from the origin, for calculation of the $R_{\emptyset}$ values.

Bands I and II showed in addition fragments of polyhedral protein with the characteristic lattice arrangement of the macromolecules, but in much smaller amounts than in the digested freeze-dried material. Band III was solely occupied by these structures and its upper boundary was reproducible in a given time in repeated experiments. The $R_{\emptyset}$ values of these protein fragments were found to be similar to those of the fastest migrating polyhedra of Heliothis zea (5.3.1.B.).

The electron micrograph of the virus suspension prior to electrophoresis (Fig. 34) shows a virus particle in an outer membrane and one without the outer membrane, both surrounded by a large amount of polyhedral protein. A particle with an outer membrane (Fig. 35) taken from band II and a particle with only an inner membrane (Fig. 36) taken from band I show association with very little polyhedral protein.

C. The granulosis virus of Heliothis armigera separated into two bands in the electrophoresis column, with a clear intermediate zone. In band II the particles were mainly within the outer membrane (Fig. 38) and in band I particles within the inner membrane predominated (Fig. 39).

The upper boundary of band II was used for measurement of the distance of migration of the virus particles enclosed in the outer membrane, and the upper boundary of band I was taken as the distance of migration of the particles contained in the inner membrane. Due to scarcity of free virus particles, this experiment was not repeated.

Electron micrographs of the starting material for electrophoresis (Fig. 37), of a particle from band II (Fig. 38) and one from band I (Fig. 39) show a convincing degree of purification.

5.3.3. $R_{\emptyset}$ values for the INCLUSION BODIES and the VIRUS PARTICLES.

The distance migrated by the virus particles or the inclusion bodies was measured from the midpoint of the origin (O) to the upper boundary of the respective band. Phenol red had a higher electrophoretic mobility than these viral elements and the distance migrated by it was taken as the distance between the midpoints of the origin (O) and the phenol red bands. The ratio of the distance of migration of the viral sample to that of phenol red gave the $R_{\emptyset}$ value for the sample.

Table 5.3.1. The $R_{\emptyset}$ values for the inclusion bodies and their virus particles at pH 8.6 and ionic strength 0.1.

Sample		Distance migrated by the sample cm	Distance migrated by the phenol red cm	$R_{\emptyset}$ value	Variations of $R_{\emptyset}$ in one or more experiments
Nuclear-polyhedra of *Colias electo*		16.7	18.4	0.91	0.90
Nuclear-polyhedra of *Heliothis zea*		8.9	12.1	0.73	0.75
Granulosis capsules of *Heliothis armigera*		13.0	14.7	0.88	-
Nuclear-polyhedrosis virus particles of *Colias electo*	In inner membrane	10.2	17.7	0.57	0.47, 0.56
	In outer membrane	13.0	17.7	0.73	0.65, 0.66
Nuclear-polyhedrosis virus particles of *Heliothis zea*	In inner membrane	9.5	17.5	0.48	0.52, 0.60, 0.61
	In outer membrane	14.0	17.5	0.80	0.84
Granulosis virus particles of *Heliothis armigera*	In inner membrane	8.7	16.1	0.54	-
	In outer membrane	11.7	16.1	0 .76	-

Fig. 30. Diagrammatic representation of the migration of the VIRUS PARTICLES in the electrophoresis column.

A. The nuclear-polyhedrosis virus of Colias electo.

B. The nuclear-polyhedrosis virus of Heliothis zea.

C. The granulosis virus of Heliothis armigera.

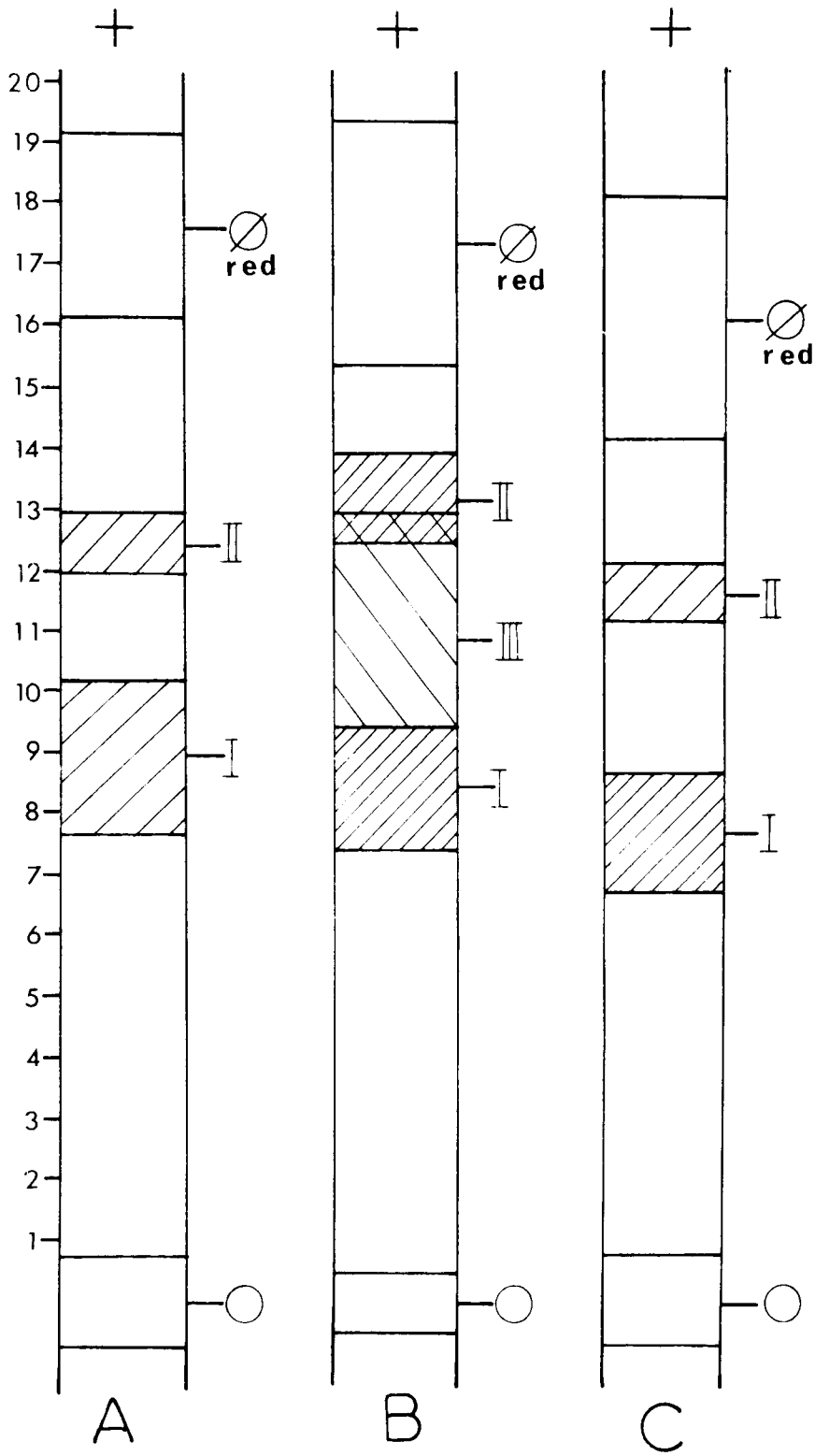
O. Origin.

Ø red. Phenol red.

I. Predominantly virus particles within the inner membrane (see text).

II. Predominantly virus particles within the outer membrane (see text).

III. Fragments of polyhedral protein matrix.

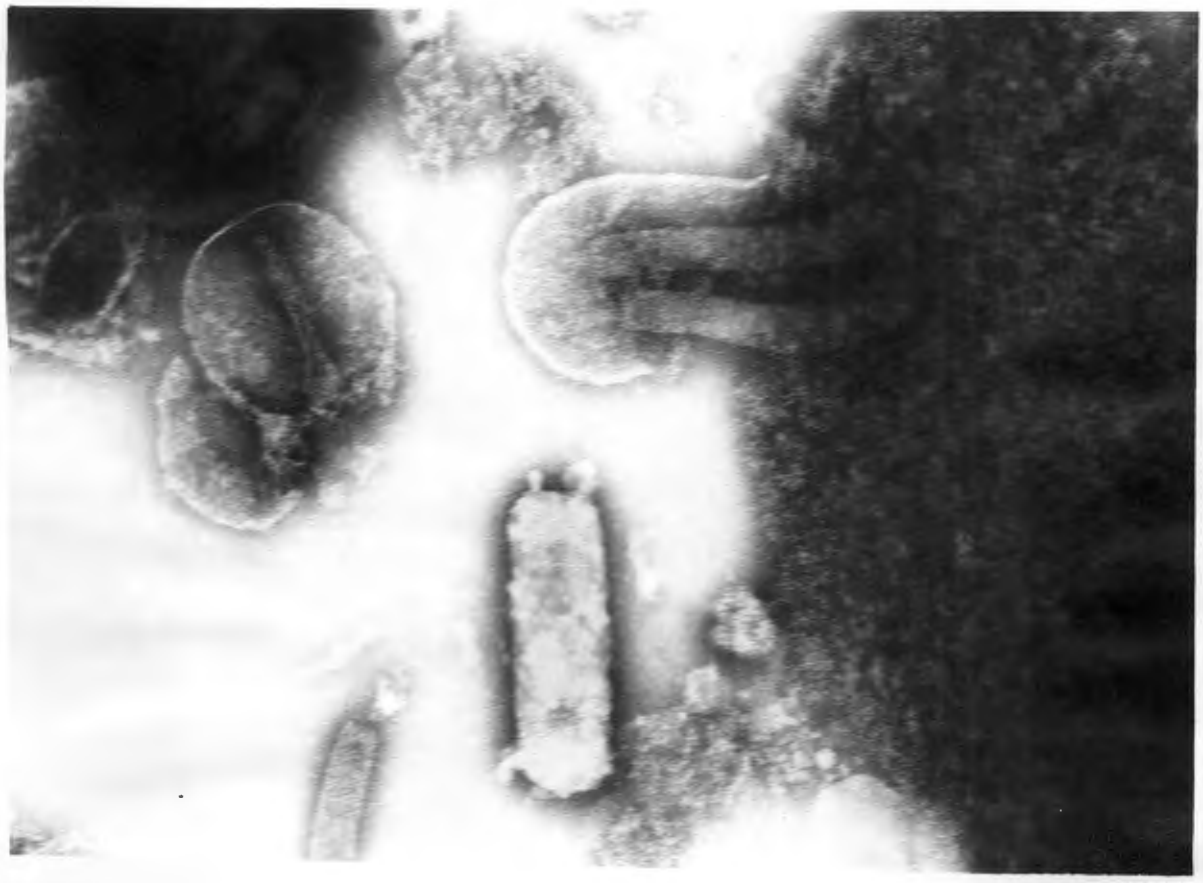

20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
red
II
I
red
II
III
I
red
II
I
A
B
C

Figs. 31-33. Nuclear-polyhedrosis virus particles of *Colias electo*. Negatively stained. Magnification X 120,000.

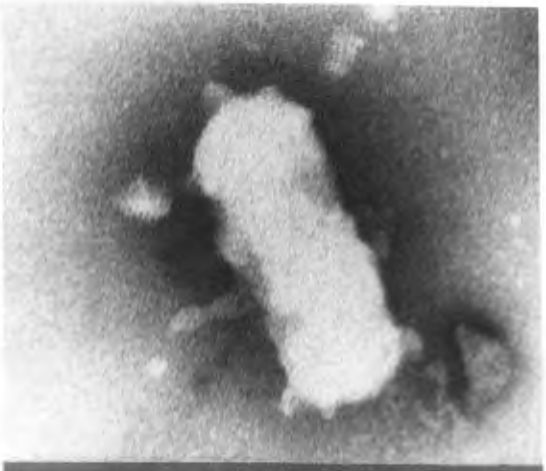
Fig. 31. Virus particles after release from the polyhedra. Before density gradient zone electrophoresis.

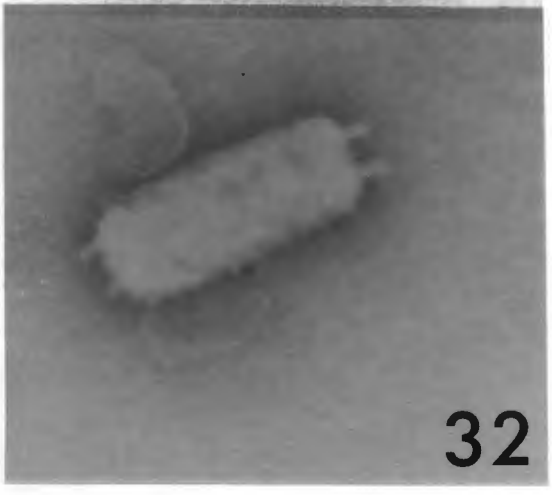
Fig. 32. Virus particles within the outer membrane recovered from band II (Fig. 30A) after density gradient zone electrophoresis.

Fig. 33. Virus particles within the inner membrane recovered from band I (Fig. 30A) after density gradient zone electrophoresis.

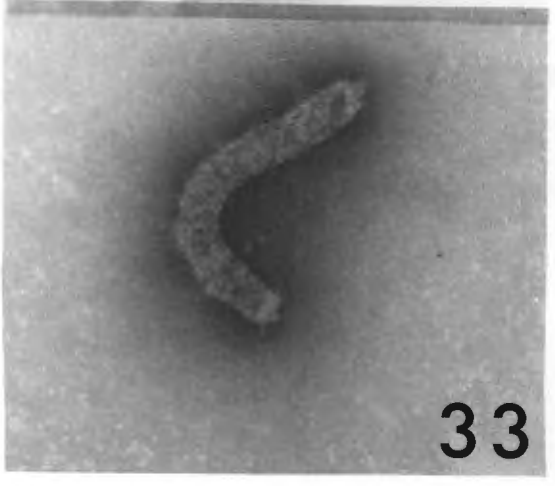


31





32



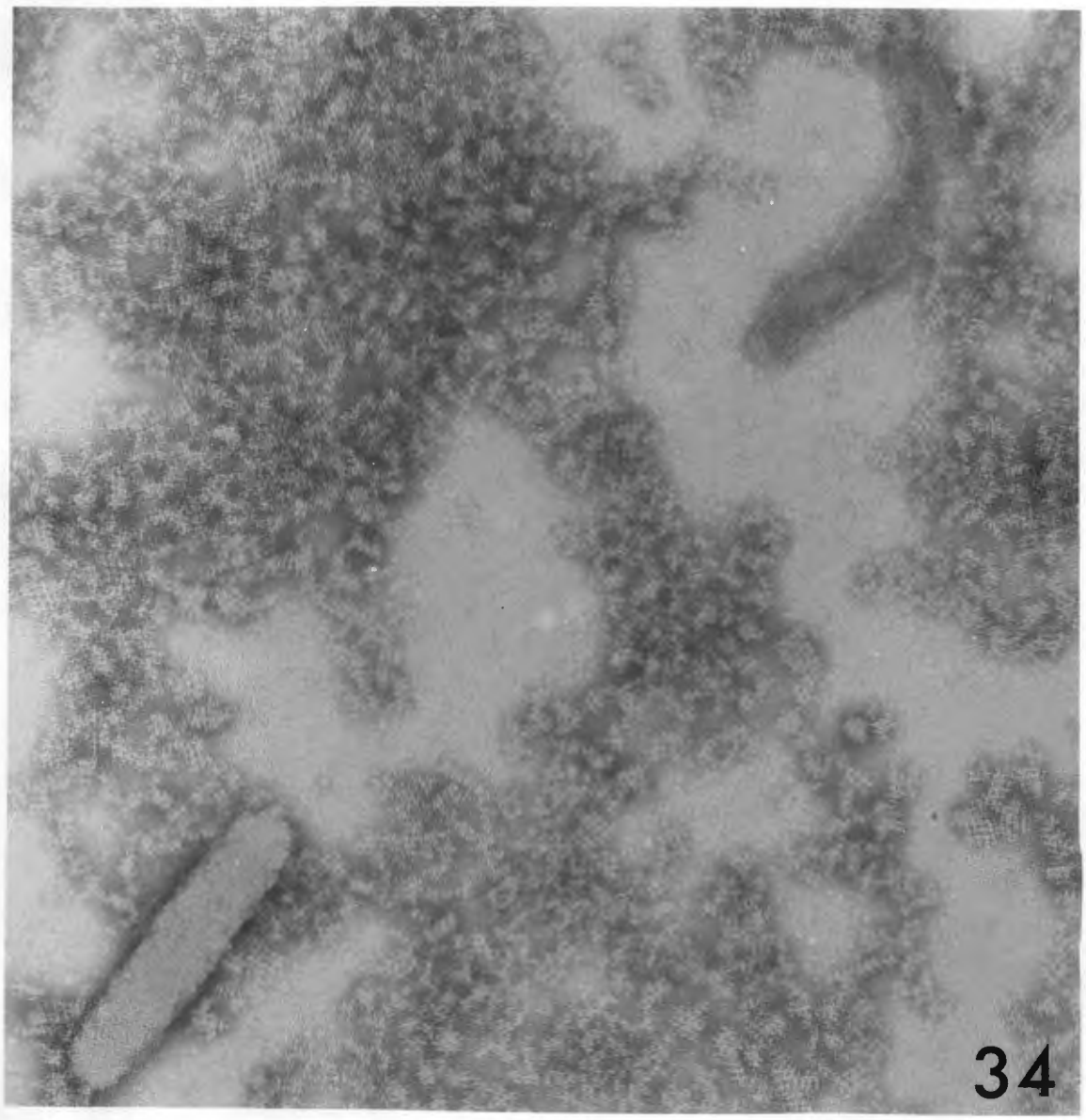
33

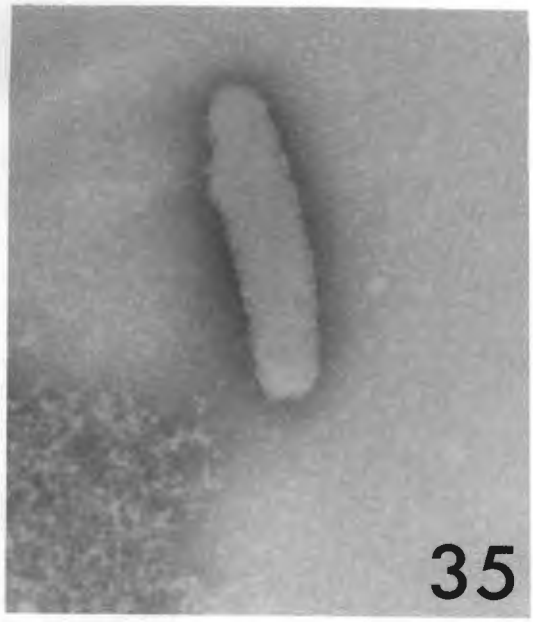
Figs. 34-36. Nuclear-polyhedrosis virus particles of Heliothis zea. Negatively stained. Magnification X 120,000.

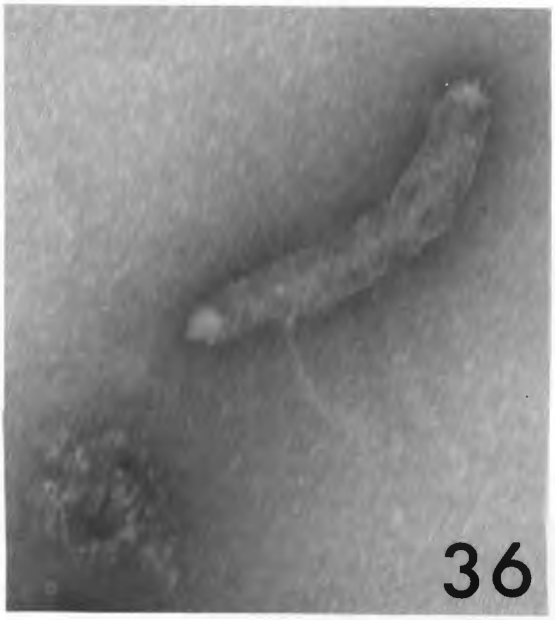
Fig. 34. Virus particles after release from the polyhedra. Before density gradient zone electrophoresis. Note the residual fragments of polyhedral protein.

Fig. 35. Virus particles within the outer membrane recovered from band II (Fig. 30B) after density gradient zone electrophoresis.

Fig. 36. Virus particle within the inner membrane recovered from band I (Fig. 30B) after density gradient zone electrophoresis.


34


35

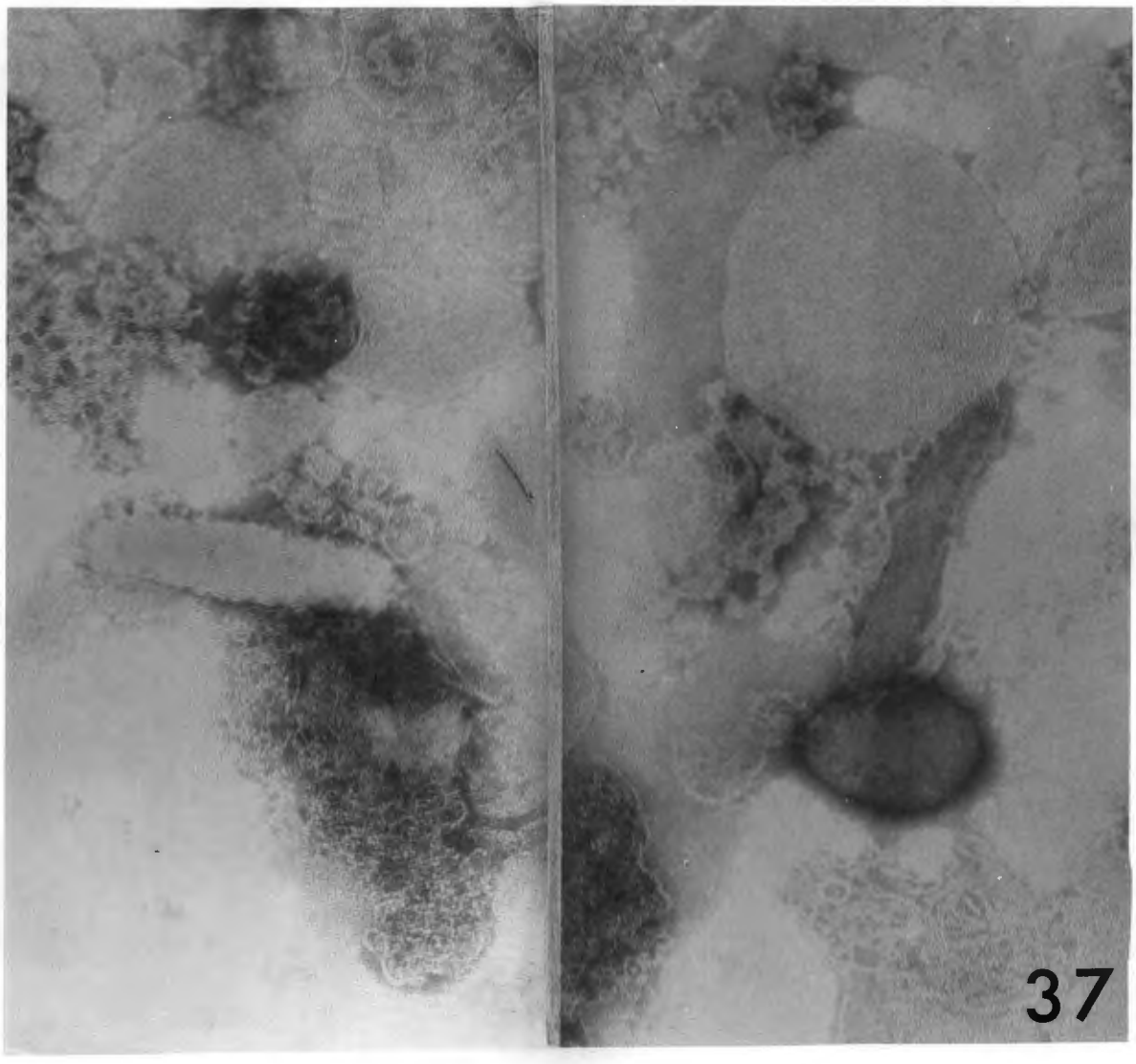

36

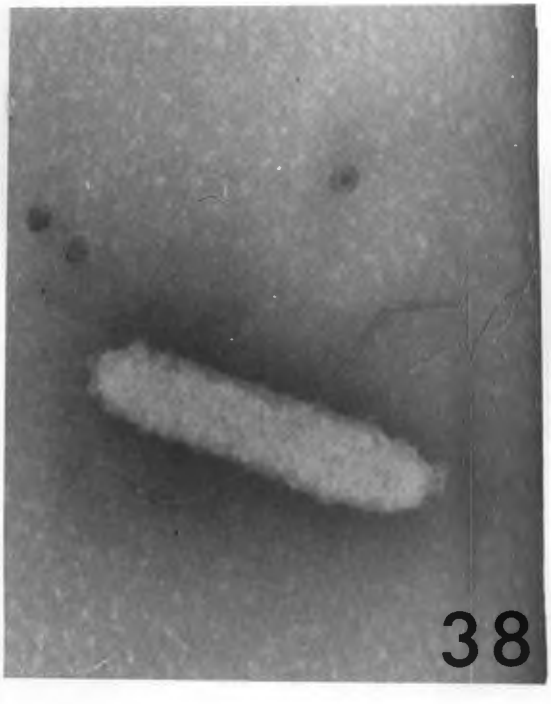
Figs. 37-39. Granulosis virus particles of Heliothis armigera. Negatively stained. Magnification X 120,000.

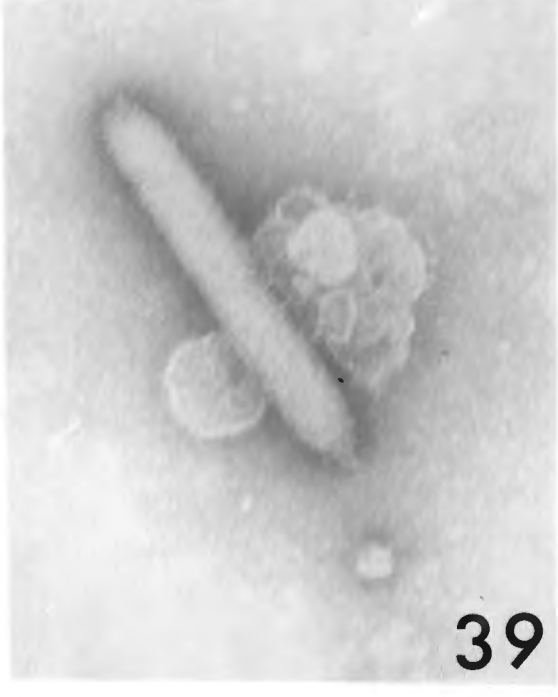
Fig. 37. Virus particles after release from the capsules. Before density gradient zone electrophoresis.

Fig. 38. Virus particle within the outer membrane recovered from band II (Fig. 30C) after density gradient zone electrophoresis.

Fig. 39. Virus particle within the inner membrane recovered from band I (Fig. 30C) after density gradient zone electrophoresis.


37


38


39

5.4. DISCUSSION

Zone electrophoresis in a sucrose density gradient proved to be a very worthwhile procedure for the further purification of the inclusion bodies and their viruses. Starting with the freeze-dried material partially purified by differential centrifugation, by treatment with an organic solvent and ultrasonication, zone electrophoresis brought about an effective separation of the inclusion bodies and virus particles from a vast amount of extraneous matter. The extent of the purification could be judged by electron microscopy of the fractions taken from the electrophoresis column.

There was a strong suggestion that the fastest moving polyhedra were those that had lost their membranes since the rod-shaped virus particles could be seen within them. The corresponding relative migration values of the apparently membrane-free polyhedra and the fragments of polyhedral protein support this suggestion. Those polyhedra wholely or partly covered with membrane appeared more opaque in electron microscopy and migrated less rapidly in the electric field. The various degrees of shedding of the polyhedral membrane seemed to offer an explanation for the varying width of the bands in which the polyhedra predominated. Lower in the column the wide opalescent band contained many aggregates, the largest of which had sufficient mass to sediment into the lowest levels of the gradient. The free polyhedra which were found in this zone

may have been released from aggregates during electrophoresis or during preparation for electron microscopy.

The free granulosis capsules formed a narrow band of homogeneous material. This was probably due to the stability of the capsular membrane which remained intact.

The $R_{\emptyset}$ values obtained for the inclusion bodies of the three different viruses were distinctive. This, however, was not the case for the respective virus particles.

Electrophoresis of the virus particles resulted in separation into two distinct, though somewhat variable, bands. In addition there was a striking tendency for the virus particles contained in the outer membrane to migrate faster than those particles that had shed this membrane. It seemed that the net negative charge on the surface of the outer membrane was greater than that on the surface of the inner membrane and for this reason electrophoresis was successful in separating them. The uppermost boundaries and the widths of the two bands tended to vary from one experiment to another. The reason for this seemed to be the variable amount of membrane that remained attached to the virus particles. Consequently, the $R_{\emptyset}$ values obtained were not characteristic for each of the viruses studied. However, density gradient zone electrophoresis was a successful method for further purification of the virus particles and for the almost complete separation of the virions enclosed in the one or the two membranes.

These experiments were performed under conditions which had been found suitable for other viruses, viz. at a pH value

of 8.6 and ionic strength of 0.1, in a sucrose gradient of which the effective range varied from 35% to 5%. It may well be shown that other conditions would favour the separation of the various components of these viruses of insect origin.

With the degree of purification achieved by the methods employed in Chapters 5 and 6, it has been possible to make a detailed study of morphology of the polyhedra, the capsules and the virus particles; and to record a number of biophysical properties of the viral elements such as the effect of organic solvents, ultrasonication and alkali digestion and the estimation of the electrophoretic mobility in terms of the mobility of phenol red represented as the $R_{\emptyset}$ value.

CHAPTER 6

MICROSCOPY OF THE INCLUSION BODIES AND THE VIRUS PARTICLES

6.1. INTRODUCTION

Polyhedral inclusion bodies are relatively large and the polyhedra are highly refractile. Accordingly, they are easily seen under the light microscope even without staining and it was as early as 1856 that they were first observed by Cornalia and Maestri. In 1872 Verson drew attention to the crystal-like structure (Morgan et al., 1955). This was confirmed by Smith and Wyckoff (1950) using electron microscopy. The highly refractile nature of the polyhedra was noted by Steinhaus (1949).

Granulosis capsules are just within the resolution of the optical microscope and although they are not as refractile as the polyhedra (Steinhaus, 1947), their granular refractile appearance also makes them visible without staining. Granulosis capsules were first observed by Paillot in 1924 using the dark field microscope but it was not until 1949 that Bergold recognized them as capsular bodies in which virus particles are occluded (Huger, 1963).

In the present study light microscopy was used to detect inclusion bodies in diseased insects and to check their integrity after each purification procedure. Since the details of their shape, size and fine structure could be

studied best by electron microscopy, staining techniques for light microscopy were not considered necessary.

Electron microscopy is essential for the detection and identification of virus particles since in the light microscope only bundles of viruses can be recognized by virtue of their Brownian motion (Paillot, 1924, in Hughes, 1950).

For electron microscopy of insect viruses heavy metal shadowing techniques were first used. The outlines of the rod-shaped nuclear-polyhedrosis viruses, many of them occurring in each polyhedron (Bergold, 1947, in Bergold, 1963a) and of the granulosis viruses, which occur singly in each capsule (Bergold, 1948, in Huger, 1963) were thus demonstrated. Bergold (1950) showed that nuclear-polyhedrosis and granulosis virus nucleoprotein are occluded in inner and outer membranes, sometimes referred to respectively as intimate and developmental membranes. Since the term 'developmental' may be misleading, as suggested by Smith and Xeros (1954b), the simpler description of 'outer' and 'inner' membranes is used throughout this thesis.

In studies of thin sections of material from diseased caterpillars, the existence of the two membranes around granulosis and nuclear-polyhedrosis viruses has been confirmed by a number of workers (Bird, 1952; Smith and Xeros, 1954b; Bergold, 1963b). While the inner membrane was shown to occlude only one virus particle in both groups of viruses, and the outer membrane of the granulosis virus occludes one virion as well, the outer membrane of the

nuclear-polyhedrosis virus may occlude one or several virus particles. The highest recorded number is 21 virus particles in a bundle (Adams and Wilcox, 1968) while up to 7 commonly occur (Morgan et al., 1956; Teakle, 1969).

Thin sections of sedimented pellets of inclusion bodies obtained by centrifugation gave further evidence of the structure of the enclosed virus particles and also showed the random distribution of the viruses in the polyhedra. The regular paracrystalline lattice of the protein molecules was also demonstrated (Morgan et al., 1955, 1956). Granulosis capsules were shown to be built in the same way (Bergold, 1963b; Huger, 1963) and so were the cytoplasmic-polyhedra (Bergold and Suter, 1959). Electron microscopy showed no components in the inclusion body other than the protein lattice and the occluded virus particles, thus confirming the findings of Bergold in 1948 (Bergold,1964) that the polyhedron consists solely of noninfective protein with a small element of infective viral material.

Negative staining revealed more details of the fine structure of insect viruses (Smith, 1967). In this way the fine structure of the inner membrane was shown (Harrap and Juniper, 1966; Teakle, 1969) and the helical structure of the nucleoprotein was made apparent by Krieg (1961, in Smith, 1967) and Koslov and Alexeenko (1967). The inner helix of granulosis viruses was demonstrated by Smith and Hills in 1962 (Smith, 1967) and by Sidor and Krstić (1969). All nuclear-polyhedrosis and granulosis viruses studied were found to contain DNA (Bergold, 1964) as a double helix (Onodera et al., 1965).

Viruses may be prepared for electron microscopy by several techniques, two of which have been used in the present study:

(a) suspensions of material negatively stained, and

(b) sections of material positively stained.

6.1.1. Negatively stained suspensions.

A. Support films. The support grids on which the virus suspension is mounted, must be coated first with a supporting film. According to Pease (1964), an ideal support film consists of light atoms, must be thin but strong and stable and adhere strongly to the grid. Its thickness should be of the order of 200 Å, transparent to electrons so that it does not reduce contrast and resolution.

Formvar (polyvinyl formal) is a plastic film which meets some of these requirements and is commonly used. This polymer is soluble in certain organic solvents and strong films are formed when the solvent evaporates. The disadvantages of this type of film are that it cannot be prepared less than 200 Å thick and that it may break or drift under the electron beam.

Evaporated carbon support films may be prepared much more thinly, down to 20 Å, and are much stronger. However, the carbon atoms are heavy and do not adhere easily to the grid. Watson (1955) developed a method of forming carbon coated grids. These are prepared in a vacuum coating unit by evaporating carbon onto grids precoated with a plastic film. The plastic substrate can then be dissolved.

Unless a very high degree of resolution is required, it is adequate to coat a thin carbon layer over the plastic film and so stabilize it (Pease, 1964).

B. Negative staining. Negative staining is achieved by surrounding a particulate object with electron dense material, so that it appears light on a dark background (Pease, 1964). This was first observed by Farrant (1954) while studying positively stained ferritin molecules. Hall (1955) was the first to try phosphotungstic acid on viruses for this purpose and to suggest its use as a method of enhancing contrast. Brenner and Horne (1959) introduced the use of potassium phosphotungstate at a neutral pH, thus preventing positive staining and enhancing the negative staining. The negative staining technique has since been used for a wide range of viruses, giving information about the structure and arrangement of subunits of the virion (Brenner et al., 1959; Huxley and Zubay, 1960; Horne and Wildy, 1963). A wide range of insect viruses have been studied by this technique. The nuclear-polyhedrosis viruses of Colias electo and Heliothis zea which are dealt with in this thesis, were also studied by the negative staining method in electron microscopy (Tripconey, 1969; Gregory et al., 1969).

The exact mechanism of negative staining is not known. Horne and Wildy (1963) and Horne (1965) suggest that because the stain dries faster than the particles, it forms a 'rigid glass' around them, thus outlining the fine surface structure. The protein mass of the intact particles is not penetrated by

the stain and is only visible on the dark background. However, it was found that some penetration of stain does occur into large crevices such as axial holes and gives the negative staining technique the additional advantage of revealing internal substructures (Horne and Wildy, 1963).

Certain conditions are essential for successful staining. The stains should be dense, amorphous and of low molecular weight so that it can become closely associated with the components of the specimen. It should be soluble and not form crystals and should not react chemically with the specimen. It should be stable under the electron beam. The embedding film should be thin and as close as possible in volume to that of the specimen (Pease, 1964). The last condition is achieved by placing on the support grid a drop of the virus suspension, either together with or prior to the same volume of stain. An even spreading of the virus-stain drop on the support grid is also essential and to obtain this a surface active agent may be added. This may assist penetration of the crevices in the specimen by the stain (Pease, 1964).

The advantages of negative staining are that it is a simple and quick technique, it reveals substructures, gives good preservation and contrast and even small amounts of virus, not necessarily pure, can be used (Horne and Wildy, 1963). The disadvantages are that it is limited to isolated particles and therefore alkali treatment to release the insect virus particles from their inclusion bodies is necessary prior to the staining. The alkali, in addition to the negative stain, may cause changes in shape and size

of the viruses as will be discussed later. While drying, distortion of the specimen may occur (Bergold, 1963b; Ponsen et al., 1964).

6.1.2. Thin sections positively stained.

In preparing thin sections, fixation, dehydration, embedding, sectioning and staining procedures are involved. Each step should suit the material to be studied. Isolated virus particles which cannot be treated individually may be packed in a sedimented pellet which is then handled like a fragment of tissue (Glauert, 1965). Entwistle and Robertson (1968) found that a dense pellet of nuclear-polyhedra presented difficulties in sectioning and preferred cutting infected tissues. However, Ponsen et al. (1964) embedded polyhedra in a gelatin block and thereafter it was sectioned successfully as a fragment of tissue.

A. Fixation. The fixative should reach all parts of the specimen, should preserve them and also give a special protection against polymerization of the embedding material (Glauert, 1965). Successful fixation of each material depends primarily on the right choice of fixative; its pH, tonicity, temperature, the duration of fixation and the specimen thickness all influence the result (Sorvall, 1965). An illustration of the importance of the choice of fixative is that osmium tetroxide dissolved one type of cytoplasmic-polyhedrosis virus while it preserved a certain nuclear-polyhedrosis virus. The reverse applied when a permanganate fixative was used (Bergold and Suter, 1959).

Osmium tetroxide (OsO_4) is the fixative longest used for electron microscopy studies of tissues but its acidity was found to cause artifacts in the specimen. To overcome this, buffered OsO_4 of a neutral pH was introduced by Palade (1952). This fixative gives good preservation of most fine structures of a cell, especially lipid containing structures but it is not so suitable for chromatin and for histochemical studies. While the fixative penetrates, extraction of some cellular material occurs simultaneously by the solvent action of the buffer. This makes an adjustment of the duration of fixation and the temperature necessary so that the undesirable effect of extraction is limited (Glauert, 1965).

Glutaraldehyde fixation was introduced by Sabatini et al. (1963) and is the best substitute for OsO_4. It has a quicker and a better capacity for penetration, it is able to preserve nucleic acids and it gives better contrast to membranes. It fails, though, to preserve lipids.

Sabatini et al. (1963) found that a primary fixation with glutaraldehyde followed by a secondary fixation of OsO_4 always gives equivalent, or better preservation and contrast than either of them alone. Nuclear-polyhedra were double-fixed in this way by Entwistle and Robertson (1968).

B. Dehydration and embedding. Glauert et al. (1956) introduced the epoxy resins which, when polymerized, are hard enough for sectioning. The most popular epoxy resin used is the formula of Epon 812 by Luft (1961). It was used, for example, to embed insect inclusion bodies by Teakle (1969). It is better than methacrylate which was

formerly used, since it is stable in the electron beam and polymerizes evenly without tension forces affecting the virus (Glauert, 1965). As Epon does not dissolve in water, dehydration of the fixed specimen should be done first in a series of ethanol solutions. As it does not dissolve in alcohol either, an intermediate solvent, 1:2 epoxy propane, miscible with both alcohol and Epon was introduced (Luft, 1961). An additional advantage of the intermediate solvent is that, being a fluid, it may be used to dilute the viscous embedding mixture for quicker and better penetration of the specimen before final embedding. It also completes dehydration.

C. Staining. Staining may be done at different stages of preparation of the specimen but to minimize distortion and to have uniform staining, it is usually performed after the thin sections have been cut (Watson, 1958a). Even then, adequate penetration of the stain may be difficult. The degree of staining of the specimen depends on the fixation, embedding, section thickness, the uptake of stain, the duration of exposure to it and its temperature and pH values. A salt of a metal of high atomic weight and number would make a good stain since it could enhance contrast by a greater degree of scattering of the electrons (Valentine, 1961; Pease, 1964).

Uranyl acetate, as first used by Watson (1958a), is reported to increase contrast of most cell structures and to have a strong affinity for nucleic acids and viruses (Valentine, 1961).

Lead stains were originally used by Watson (1958b) in the form of alkaline lead hydroxide and lead acetate. An improvement introduced by Reynolds (1963) is lead citrate which he found to be most useful since contamination by crystals formed under exposure to CO_2 in the air occurred only after 30 min, the citrate serving as a chelating agent. Lead stains enhance contrast of specimens generally and combine with nucleic acids and cytomembranes better than uranyl acetate (Valentine, 1961). According to Watson (1958b), lead stains have an affinity for many cellular structures.

The combination of the two stains, lead citrate followed by uranyl acetate, was found to give the best contrast by Pease (1964). The double staining technique has been used on nuclear-polyhedrosis viruses (Entwistle and Robertson, 1968), but lead citrate was used solely for staining granulosis viruses (Tanada and Leutenegger, 1968).

6.2. MATERIALS AND METHODS

6.2.1. Light microscopy.

For initial detection of inclusion bodies, drops or smears of haemolymph from infected insects were examined. In subsequent isolation and purification procedures, sedimented pellets of inclusion bodies resuspended in water were used. Specimens were examined under a Cooke, Troughton and Simms microscope at magnifications X 120 and X 480.

6.2.2. Electron microscopy.

6.2.2.1. Specimen support grids.

These were copper grids of 2.3 mm diameter and 200 mesh/inch which were used uncoated for mounting thin sections. For negatively stained preparations the support films were placed on the matt side of the grid.

6.2.2.2. Support films.

A. Formvar powder (Ladd Research Industries) was dissolved in ethylene dichloride (May and Baker) solutions ranging from 0.10 to 0.25%. By experiment the best concentration was found to be 0.18% (w/v); thereafter a stock solution of 1.8% (w/v) was prepared, stored in a dark bottle at 4°C and diluted in ethylene dichloride, 1:10, before use. The solution was then cast on glass according to the method of Drummond (1950). A glass slide, 1 x 3 inches, was dipped into a staining jar filled with the formvar solution. It was then held in the ethylene dichloride vapour for a few seconds to avoid rapid cooling and consequent water condensation and was dried by holding it vertically on a filter paper. A uniform film thickness was thus obtained. The slide was then dipped into distilled water at an angle of 45°. The film stripped off the glass by surface tension and floated on the water and could be seen by reflected light. The support grids were then placed on the film, matt side down, and were picked up on a filter paper and allowed to dry.

B. Evaporated carbon film (Bradley, 1965) was prepared in an Edwards model 12 E 2 vacuum coating unit. The formvar coated grids were placed in a petri dish at a distance of 10 cm from the carbon electrodes, formvar side up. The chamber was evacuated and a current of 90 amp at 10 v passed for 2-4 sec. Sparking between the carbon rods resulted in evaporation and carbon atoms were deposited on the grids in a thin uniform layer. The amount of evaporated carbon was estimated by placing a piece of white porcelain with a drop of oil on it near the grids. The difference between the white area under the oil and the darker one around it indicated the degree of density of the deposit. Films of about 100 Å were formed.

After the grids were coated with formvar and carbon they were stored in a vacuum desiccator which prevented the copper from expanding and shrinking as a result of changes in temperature and humidity. Before use the coated grids were rinsed in carbon tetrachloride (CCl_4) to remove grease and were then allowed to dry (Valentine, 1961).

6.2.2.3. Negative staining.

A. The stain used was a modification of that of Brenner and Horne (1959). 2% phosphotungstate ($2Na_3PO_4.24WO_3+H_2O$) (British Drug House) was dissolved in distilled water and the pH adjusted with 1 N potassium hydroxide (KOH) to a range of different values between pH 6.0 and 7.0. A pH of 6.0 was found to be most suitable. After 3 hr the pH was again adjusted and the solution stored at room temperature. The container was not disturbed and

the stain used was taken from the upper layer, so ensuring that undissolved crystals of potassium phosphotungstate were not incorporated.

B. The 'drop method' (Pease, 1964) was used to mount the virus suspension on the grid. Sedimented pellets of inclusion bodies and the viruses were prepared as described in 4.2. The pellets were then resuspended in a drop of distilled water containing 0.005% (w/v) bovine albumin powder (Armour Pharmaceutical Company) which served as a surface active agent (Horne, 1965) and which, in such a low concentration, did not obscure the picture in the electron microscope. Fine pasteur pipettes were used to place a droplet of the negative stain on a microscope slide. A droplet of pellet suspensions with bovine albumin of approximately the same volume was mixed with it and applied onto the grid which was held by fine forceps. Excess fluid was removed from the edge of the grid with a pipette and by absorbing fluid from between the blades of the forceps with a filter paper. The grids were dried in air at room temperature. If the pellet to be studied was very small, a droplet of the specimen suspended with bovine albumin was first applied on the grid to avoid dilution. After allowing it to dry, it was exposed to a droplet of the stain for about 1 min.

6.2.2.4. Thin sections.

A. Embedding pellets in agar. The inclusion bodies were embedded in agar according to the method of Kellenberger

et al. (1958). The two polyhedra strains and the granulosis capsules were each centrifuged at a speed of 20,000 rpm for 30 min. The pellet formed was then loosened and a small drop of melted 2% Difco agar mixed with it. The mixture was poured onto a microscope slide and allowed to gel. Blocks of about 1 mm^3 were cut and treated thereafter as a fragment of tissue. This procedure of embedding the pellets in agar was found to be necessary because the pellets obtained at the above mentioned speed were not compact enough and exposure to higher speeds damaged the inclusion bodies. Each block of inclusion bodies in agar was fixed, dehydrated and embedded in epon without transferring the specimen from the glass container.

B. Fixation.

Buffered glutaraldehyde fixative (Sabatini et al., 1963).

(a) Glutaraldehyde aqueous stock solution, 25% (Baird and Tatlock). This solution was kept at 4°C.

(b) Phosphate buffer stock solution, 0.15 M.

$NaH_2PO_4.H_2O$	5.85 g
Ha_2HPO_4	15.25 g
Distilled water to	1 litre

Before use, (a) was diluted 1:5 in (b) to give a final concentration of 5% glutaraldehyde and a pH of 7.3.

The fixation was carried out at 4°C for 4 hours as 16 hours was found to give less satisfactory resolution. The specimen was washed in three changes of phosphate buffer and left overnight in the buffer. This thorough washing was necessary before the secondary fixation with OsO_4.

Buffered osmium tetroxide (OsO_4) fixative, 1% (Palade, 1952) (details in Sorvall, 1965).

(a) Veronal-acetate buffer stock solution, 0.28 M.

Sodium veronal	2.88 g
Sodium acetate (anhydrous)	1.15 g
Distilled water to	100 ml

(b) 0.1 N HCl.

Concentrated HCl (density 1.186 g/ml)	9.1 ml
Distilled water to	1 litre

(c) OsO_4 stock solution, 2%.

Crystalline OsO_4 (British Drug House)	1 g
Distilled water to	50 ml

This solution was kept in a dark bottle not longer than 3 days.

The fixative was made up just before use by mixing:

6.50 ml of solution (a)

5.50 ml of solution (b) to give a pH of 7.3

4.25 ml distilled water

16.25 ml of solution (c) to give a final concentration of 1% OsO_4.

Fixation and all the manipulation with OsO_4 were performed in a fume cupboard. The specimens in the bottles were covered with fixative, the bottles stoppered and left at 4°C for 90 min for fixation. The specimens were then rinsed in several quick changes of veronal-acetate buffer.

C. Dehydration. This was done through a series of ethanol solutions of increasing concentrations (Analar, Hopkins and Williams) and an intermediate solvent, 1:2 epoxy propane (British Drug House) as follows:

35% ethanol	10 min at 4°C
75% ethanol	10 min at 4°C
96% ethanol (2 changes)	15 min at room temperature
100% ethanol (3 changes)	30 min at room temperature
1:2 epoxy propane (3 changes)	10 min at room temperature

D. Embedding. The Epon mixture was made according to Luft (1961). The reagents used are marketed by Ernest F. Fullam Inc. and were mixed in the following ratios:

(a) Soft Epon mixture.

Dodecenyl succinic anhydride (DDSA)	12.0 ml
Epon 812	7.5 ml

(b) Hard Epon mixture.

Methyl nadic anhydride (MNA)	6.6 ml
Epon 812	7.5 ml

(a) was mixed with equal amount of (b) and 1.5% of the accelerator, 2, 4, 6-dimethylaminomethyl-phenol (DMP-30) was added. Calibrated syringes were used to measure the reagents and glass rods were used to mix the ingredients thoroughly. All the equipment was dried before use in an oven at 60°C. The final Epon preparation was mixed with 1:2 epoxy propane in equal volumes and poured over the specimens. Infiltration was allowed to take place at room temperature in open bottles in the fume cupboard so that excess epoxy propane could evaporate. The specimens were then rolled on filter paper to get rid of residual epoxy propane and transferred to previously dried gelatin capsules No. 00 filled with undiluted Epon mixture. The specimens were allowed to sink through the Epon mixture to the bottom of the gelatin capsules. Specimens were kept at 60°C for

2 weeks to allow polymerization to take place; blocks that had polymerized for less than 2 weeks were found to be too soft.

E. Sectioning. The gelatin capsules were removed from the Epon blocks by soaking in warm water. The end of the block containing the specimen was trimmed to the shape of a truncated pyramid in an LKB pyramitome. For sectioning the blocks, glass knives were cut in a LKB knife maker at 45° and a Reichert ultramicrotome model OM U2 was used to get the requisite thin section. In this process the knife was positioned at a 7° angle to the block. The sections had a silver colour, indicating a thickness of approximately 600-900 Å (Sorvall, 1965). Chloroform vapour was sometimes used to flatten the sections which were picked up by grids from the water using a vertical approach. Uncoated 200 mesh/inch copper grids were used. When mounted, the specimens were allowed to dry on filter paper.

F. Staining. Uranyl acetate (May and Baker) stain was made up to 1% (w/v) in distilled water and shaken vigorously in a mechanical shaker for 10 min. The solution, pH 4.7, was allowed to stand for 24 hr to allow precipitation of solids including coarse particles of stain. The upper layer of the solution was used for staining and drops were placed on a piece of dental wax and covered with a petri dish to avoid dust contamination. The grids, with the specimen facing the stain, were floated on the drops. Optimal staining time was found to be 4 min. The staining was followed by rapid rinses in three vessels of distilled water

and the grids were dried on a filter paper.

Lead citrate was freshly made up according to Venable and Coggeshall (1965).

Lead citrate (Hopkins and Williams)	20 mg
NaOH	40 mg
Distilled water	10 ml

The preparation was shaken vigorously in a tightly closed bottle. This gave a solution of 0.2% lead citrate in 0.1 N HaOH of pH 12.4. The grids were exposed to the lead citrate solution for 10 sec and subsequently rinsed and dried as described for uranyl acetate. This period of 10 sec was found to be the optimal time of exposure to the lead citrate stain after 4 min staining in uranyl acetate.

6.2.2.5. The Electron Microscope.

Siemens Elmiskop 1 A electron microscope was used. Examinations were made at 80 kv.

6.2.2.6. Photography.

The observations were recorded on Ilford photographic plates N. 40 or N. 50.

It was decided to print the electron micrographs on a Kodagraph P 84 light weight projection paper. Since this paper was not available in different grades, contrast was not always as good as that on a variety of harder papers.

6.3. RESULTS

6.3.1. Characteristics of the nuclear-polyhedrosis viruses of Colias electo and Heliothis zea.

The following findings apply to both viruses, unless otherwise stated.

A. The polyhedra. The polyhedra, when observed under the light microscope, appeared highly refractile, with a green colour, distinguishing them from bacteria and uric acid crystals which may resemble them in shape and size. While both had an hexagonal outline, Colias electo polyhedra could be distinguished from the smaller polyhedra of Heliothis zea by the difference in size.

Under the electron microscope, the majority of the polyhedra had irregular hexagonal outline with rounded corners (Figs. 40; 53). Differences in size could also be measured. The mean size of randomly chosen polyhedra and the standard deviations were calculated. Dimensions of the hexagon were measured in two ways; the distance from vertex to opposite vertex and the distance from side to opposite side were taken and the three measurements obtained in each way were averaged. These results are set out in Table 6.3.1.

Each polyhedron was surrounded by a thin membrane (Figs. 40; 41; 55). In thin sections the membrane had a greater affinity for the stain than the inclusion body (Figs. 42; 43; 44P; 56).

The regular cubic paracrystalline lattice of the protein molecules of the polyhedra was demonstrated (Figs. 44P; 54; 55).

Following treatment with alkali, the polyhedral protein crystal broke up into fragments that showed the paracrystalline lattice and as digestion advanced these fragments separated into smaller pieces. After alkali treatment of the polyhedra many more fragments of the protein lattice were associated with Heliothis zea preparations (Figs. 58; 60; 61; 62; 63) than with Colias electo preparations (Fig. 47P).

B. The virus particles within the polyhedra. As a general rule, the virus particles were found to be embedded in the polyhedral protein and were distributed randomly. In the nuclear-polyhedra of Heliothis zea the virus particles appeared singly (Fig. 56), whereas in the inclusion bodies of Colias electo they were present either singly or in bundles (Figs. 42; 43; 44) and in each bundle 2-8 virus particles occluded by a common outer membrane were observed. As seen in thin sections, each Colias electo polyhedron occluded either only single viruses (Fig. 44S) or combinations of both single viruses and bundles, but predominantly bundles (Figs. 42; 43). The same was seen in negatively stained preparations of the Colias electo polyhedra, but the distinction was made by width as the single viruses (Fig. 45) were narrower than the bundles (Fig. 46) (see Table 6.3.1.). Within each Colias electo polyhedron the length of the virus particles tended to be very uniform, but the width of the bundles was determined by the number of virus particles inside them. In each Heliothis zea polyhedron, where the virus particles appeared

only singly, both their length and width were remarkably uniform.

Measurement of the virus particles inside the polyhedra of both species was done mainly in negatively stained suspensions of polyhedra as this was found to be difficult in thin sections due to the random distribution of the virus particles. For example, longitudinal sections of virus rods were seen very rarely (Fig. 44L) while cross sections, although more common, were also rare. The width of a virus or a virus bundle within the outer membrane both inside and outside the polyhedra was smaller in thin sections than in suspensions of viruses (Table 6.3.1.). The same may be said of the viruses within the inner membrane.

In both species, when the inclusion body was only partially digested the virus particles were exposed but they were still held by the matrix of the residual polyhedral protein (Figs. 45; 46).

C. The virus particles within the outer membrane.

At the next stage of degradation resulting from alkali treatment the virus or the viruses within their outer membrane were released completely from the inclusion body. In suspensions the virus particles within the outer membrane appeared opaque (Figs. 47A, D; 57). Likewise, in thin sections made through the membrane or just above it, no internal structure was revealed (Figs. 44S1, P1; 56 arrow). In both species the outer membrane was thick and built up of two layers (Figs. 44L; 60) and there was a space between it and the occluded virus (Figs. 42; 43; 44; 56). Measurements of the length and width of bundles or single virus particles

within the outer membrane whether occluded in or liberated from the polyhedra showed no significant difference and they were fairly constant (Table 6.3.1.). When in a bundle they were packed tightly (Figs. 42; 43).

The nuclear-polyhedrosis virus particles of Colias electo within the outer membrane were not curved and always appeared straight although the outer surface showed wave-like convolutions of the side walls. At the ends projections were observed; the single occluded viruses showed a projection on one (Fig. 48S) or both ends. For each of the occluded viruses in a bundle, one projection on one or both extremities was seen, e.g. two (Fig. 48B), three (Fig. 47A) and four (Fig. 50D). A protrusion of the internal material through the projection was seen in Fig. 50D and there was a looped projection at one end in Fig. 49.

The virus particles of Heliothis zea within the outer membrane appeared straight when inside the polyhedra (Figs. 55; 56) but when the latter were partially or completely dissolved, some of the viruses had a horseshoe or curved appearance (Figs. 57; 58; 59). No projections or protrusions were observed on the straight particles but they were always seen on the horseshoe-like ones. The projections were seen at both ends either as straight projections (Fig. 59) or when bent and they touched each other, a loop-like appearance resulted (Fig. 57). When the projections appeared at one end only they appeared looped (Fig. 58) or straight.

In a further stage of degradation in alkali the virus particles shedded the outer membrane. The partially

dissolved membrane of Colias electo virus formed irregular folded arborescent strands (Fig. 47C) but remained temporarily attached to the virus particles at both ends (Fig. 51).

The outer membrane of the nuclear-polyhedrosis virus of Heliothis zea ruptured in a different way from that of Colias electo. In most cases it ruptured in the centre forming two spheres of roughly equal size (Fig. 60A, B). However, sometimes it opened at one end to allow the escape of the virus (Fig. 60C).

D. The virus particles within the inner membrane. The nuclear-polyhedrosis virus of Colias electo appeared in straight or curved form with either form sometimes showing transverse lines (Fig. 47C, V). Some virus particles appeared to be compressed from both ends (Fig. 47M). The nuclear-polyhedrosis virus of Heliothis zea also appeared compressed but twisted (Fig. 61). This suggestion of compression was also conveyed by the appearance of the particle when seen within the outer membrane (Figs. 47A, D; 57). When still inside the outer membrane but while emerging from it, the virus particles of Colias electo were straight (Fig. 47C) and those coming from within the same membrane were uniform in size. But, when free, due to the bending, compression and twisting, the length of the virus particles showed obvious variation. The width of the virus particles of both species within the inner membrane was remarkably constant as can be seen in thin sections (Figs. 42; 43; 44; 56) as well as in negatively stained virus suspensions (Figs. 47; 51; 61). The length of the virus particles

within the outer membrane was less than that of the freed infective units because the latter were no longer compressed.

The two ends of the virus particles were distinct in density and shape. 'Claw-setting' was observed at both ends but an additional 'nipple' was very often seen at one of these ends (Figs. 47V; 50M; 60A; 61). In Fig. 60A the nipple appeared like three claw-setting rings tapering towards the end. The claw-setting was observed on both ends of empty inner membranes (Figs. 47E; 62F) but the nipple was seldom seen on the empty structures (Fig. 63).

The inner membrane was built of capsomeres, but their presence was more easily detected on the outline of the walls of the empty membranes (Figs. 47E; 51 arrows; 62; 63). Empty membranes showed no transverse lines like those seen when the virus was within the inner membrane, and were shorter than the full membranes (Fig. 47E).

When partially dissolved the inner membrane exposed the nucleoprotein helix (Figs. 52; 64A). Upon release the latter uncoiled showing two bands which formed straight or looped structures (Figs. 52 arrow; 60C; 64B).

Fig. 40. Colias electo polyhedra showing the hexagonal outline with rounded corners, the surrounding membrane (arrows) and the occluded rod-shaped virus particles. Negatively stained. Magnification X 100,000.

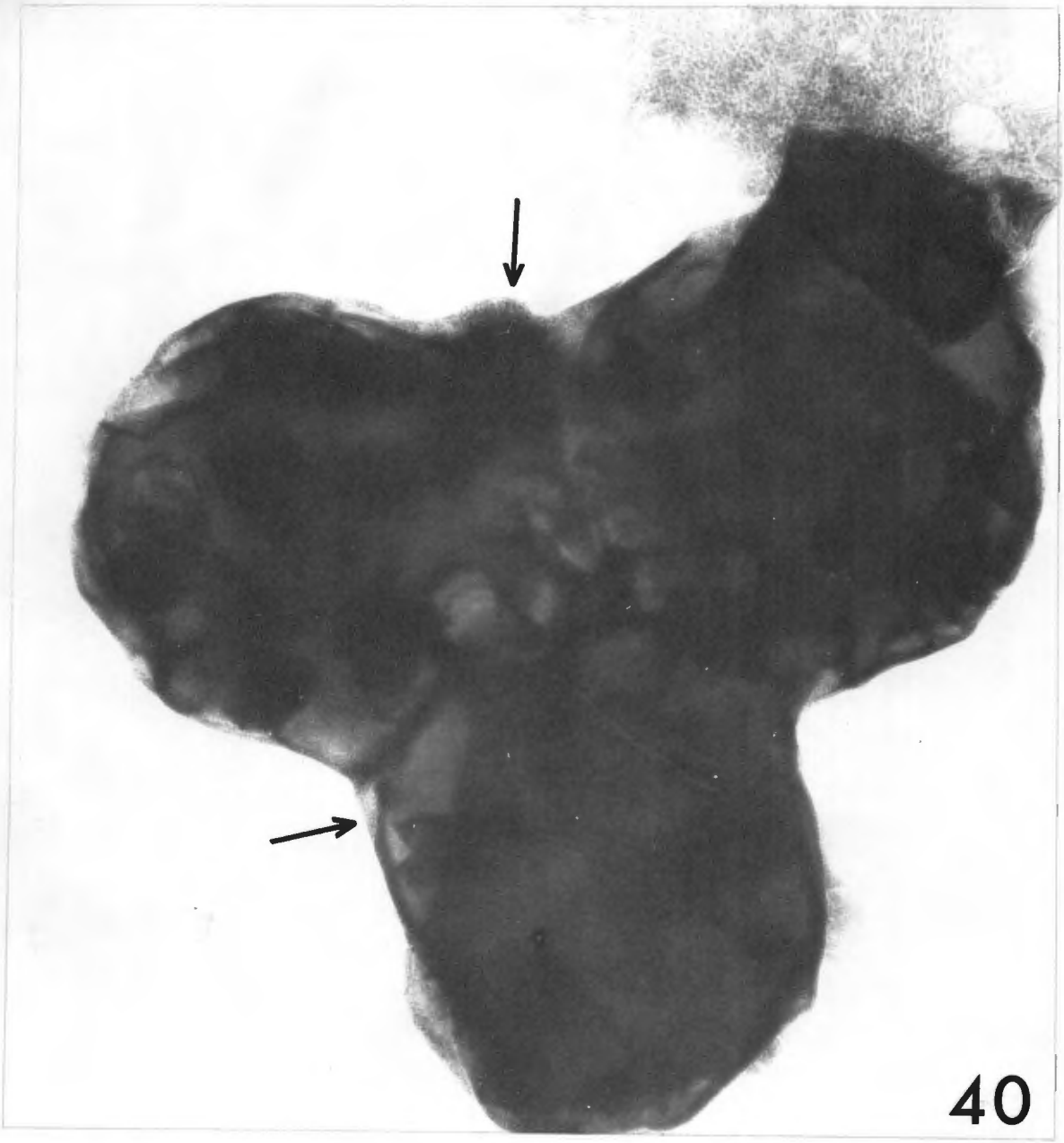
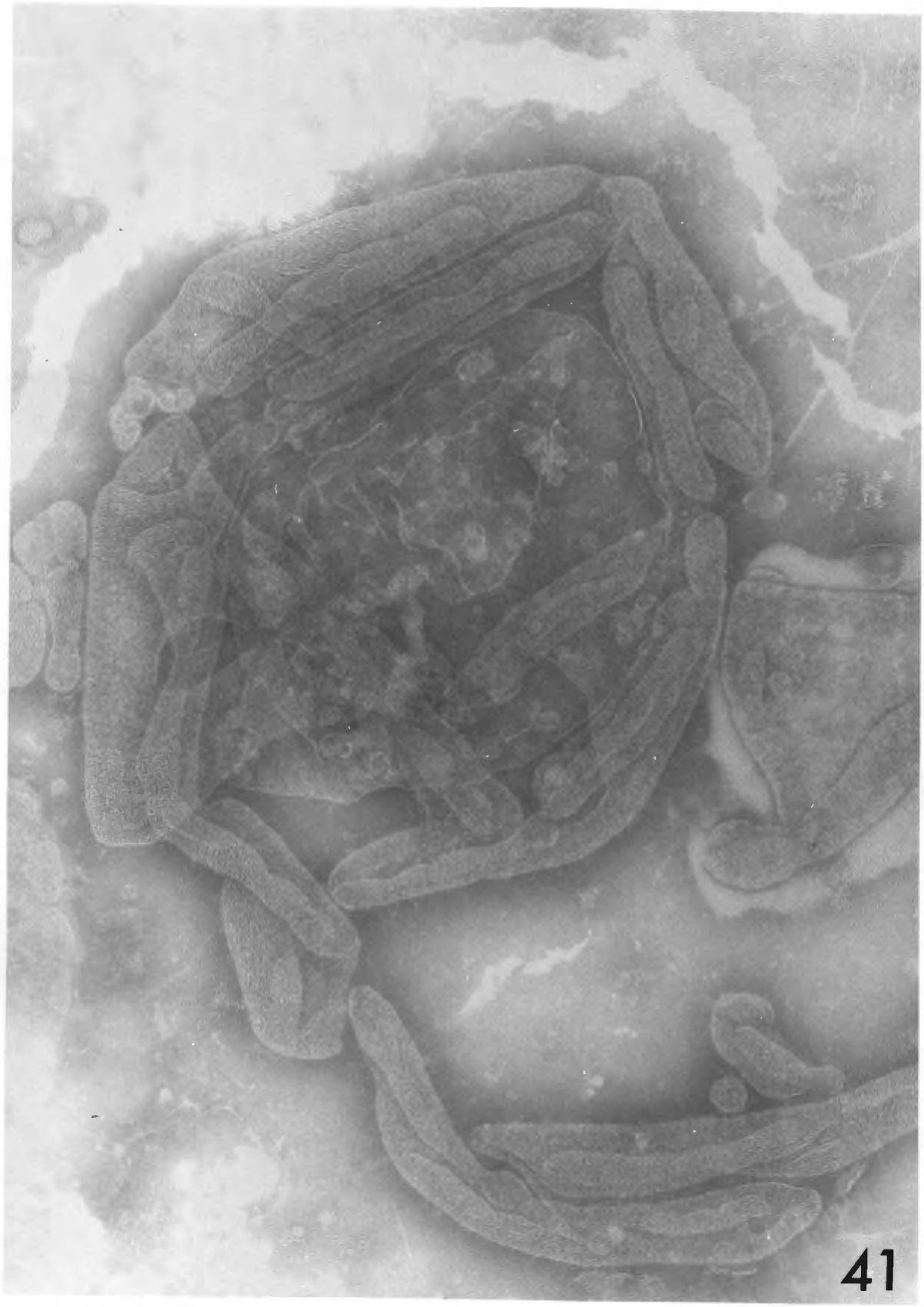

40

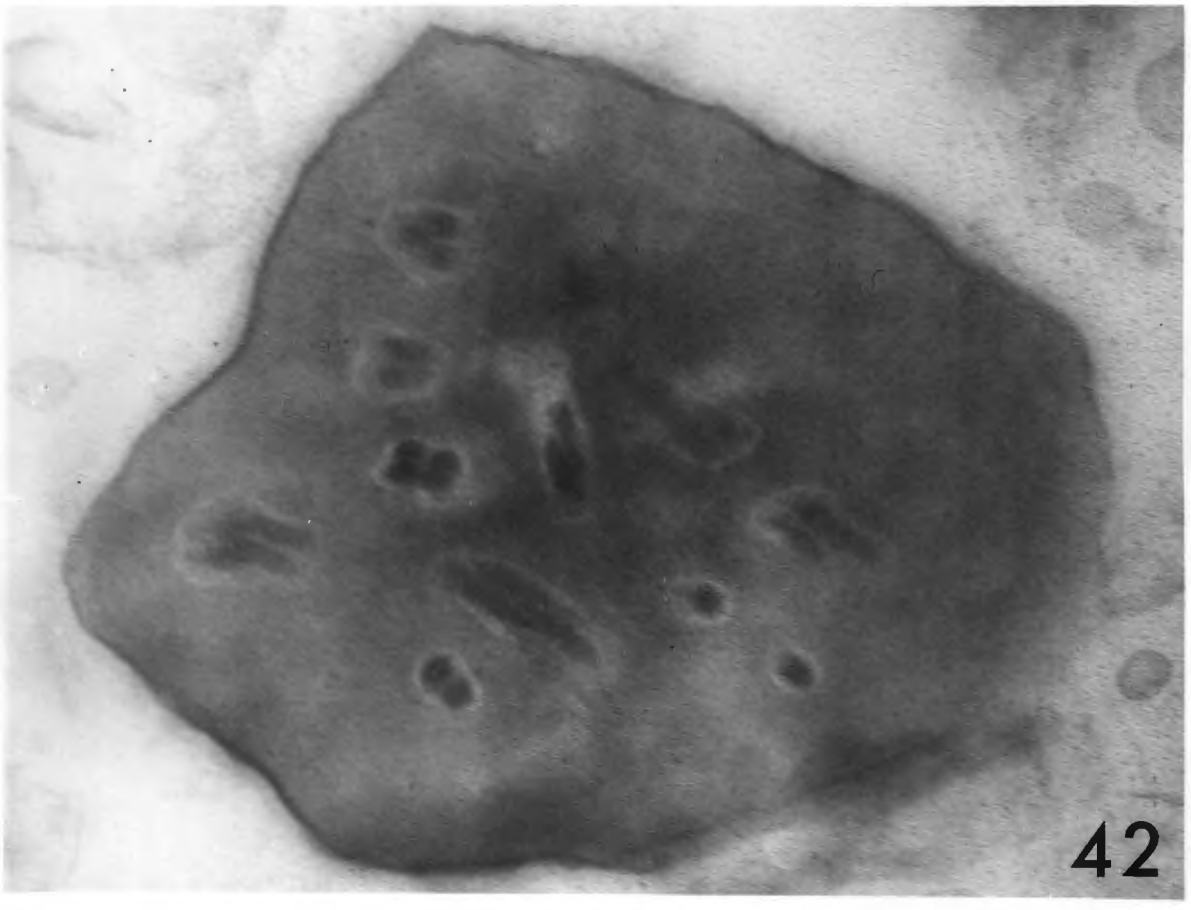
Fig. 41. Residual folded membrane of a Colias electo polyhedron after alkali treatment. Negatively stained. Magnification X 120,000.


41

Figs. 42-43. Thin sections of *Colias electo* polyhedra showing virus particles occluded either singly or in bundles and randomly distributed. Note the deeper staining of the polyhedral membrane. Magnification X 120,000.

Fig. 42. Note that the number of virus particles in each outer membrane varies from 1 to 4.

Fig. 43. Note the absence of single virus particles and the occurrence of a bundle containing 8 virus particles within the outer membrane.


42

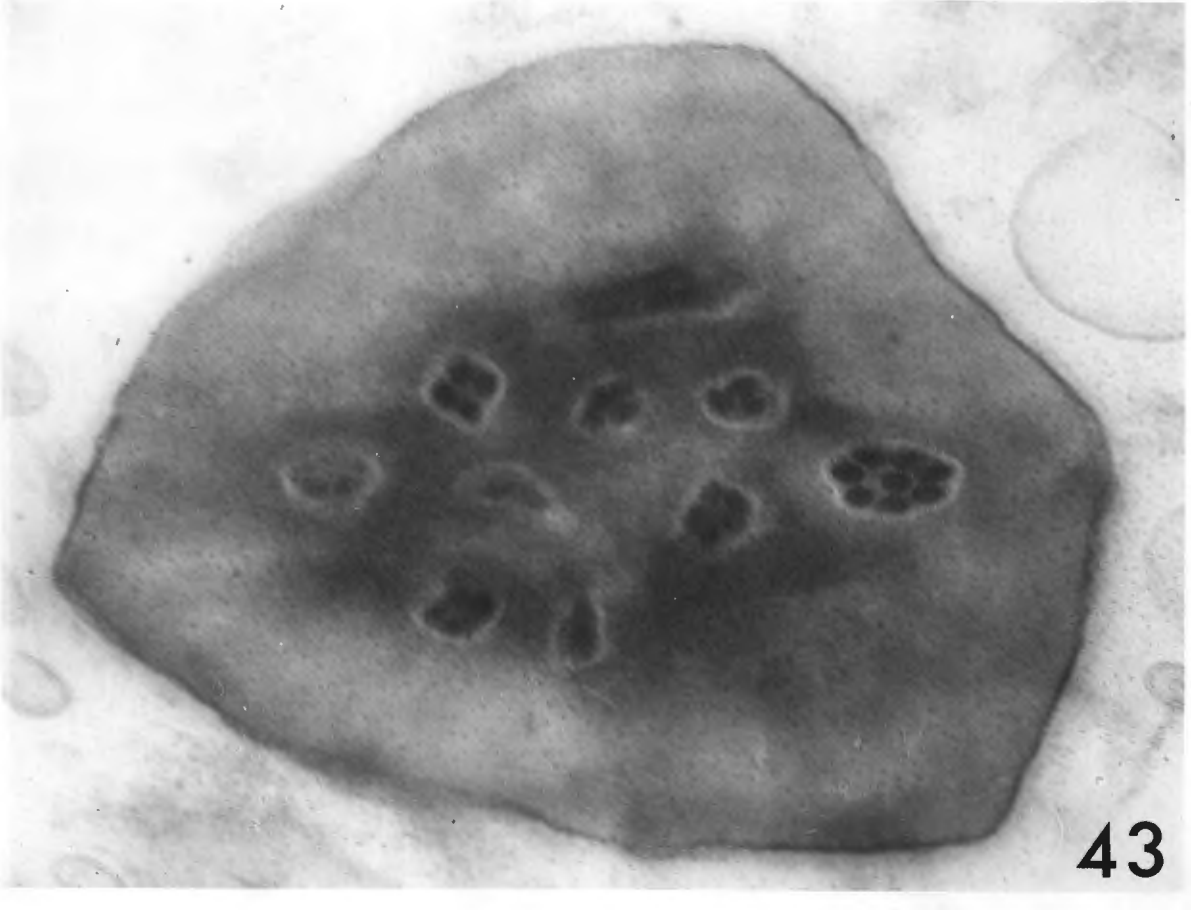
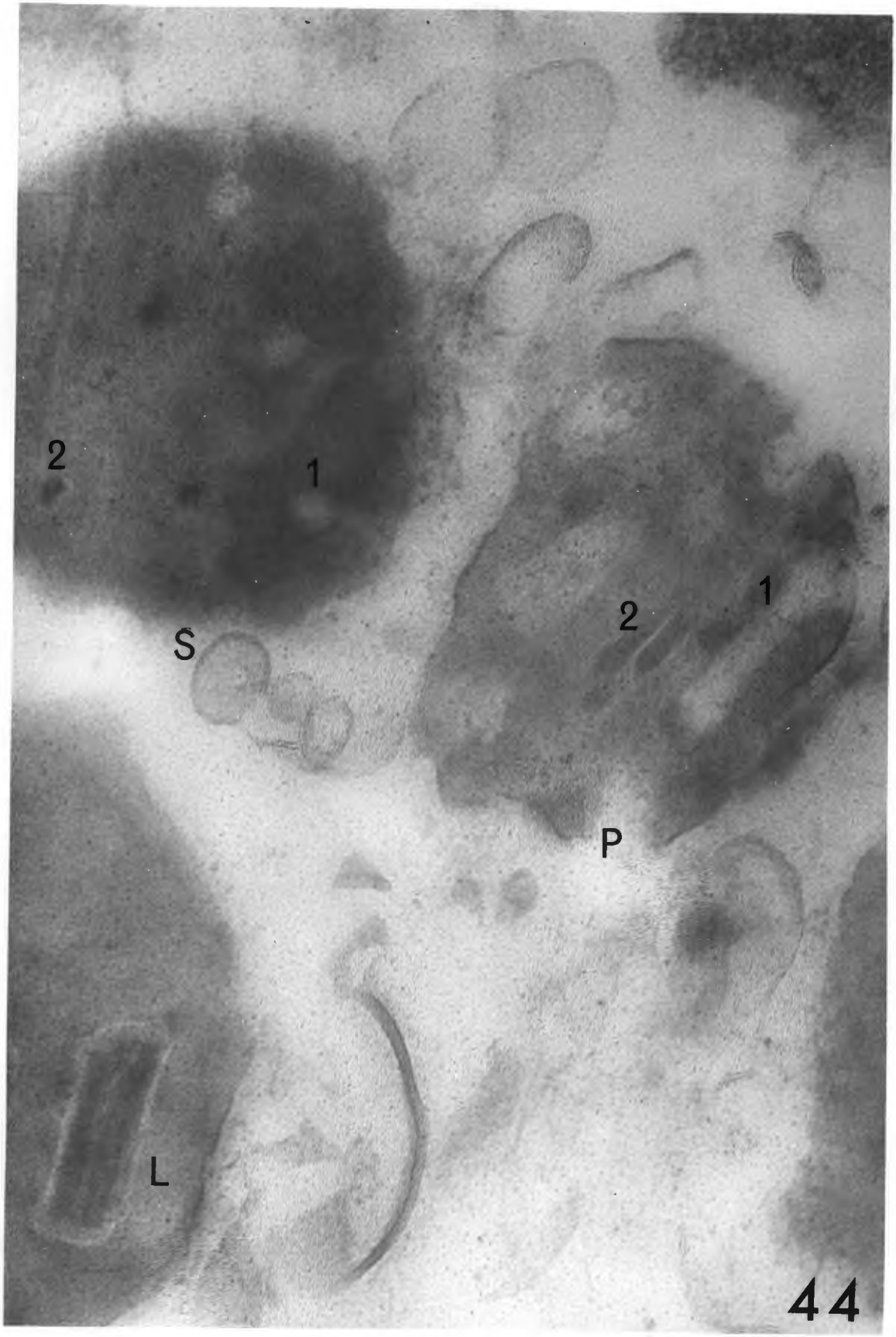

43

Fig. 44. Thin section of Colias electo polyhedra. Magnification X 120,000.

S. A polyhedron occluding only single virus particles.

P. A polyhedron occluding bundles of virus particles and showing the lattice arrangement of the protein molecules. Note the different appearance of virus particles sectioned through the outer membrane (1) compared with those sectioned through both the outer and inner membrane (2).

L. A virus bundle longitudinally sectioned. Note the double outer membrane.

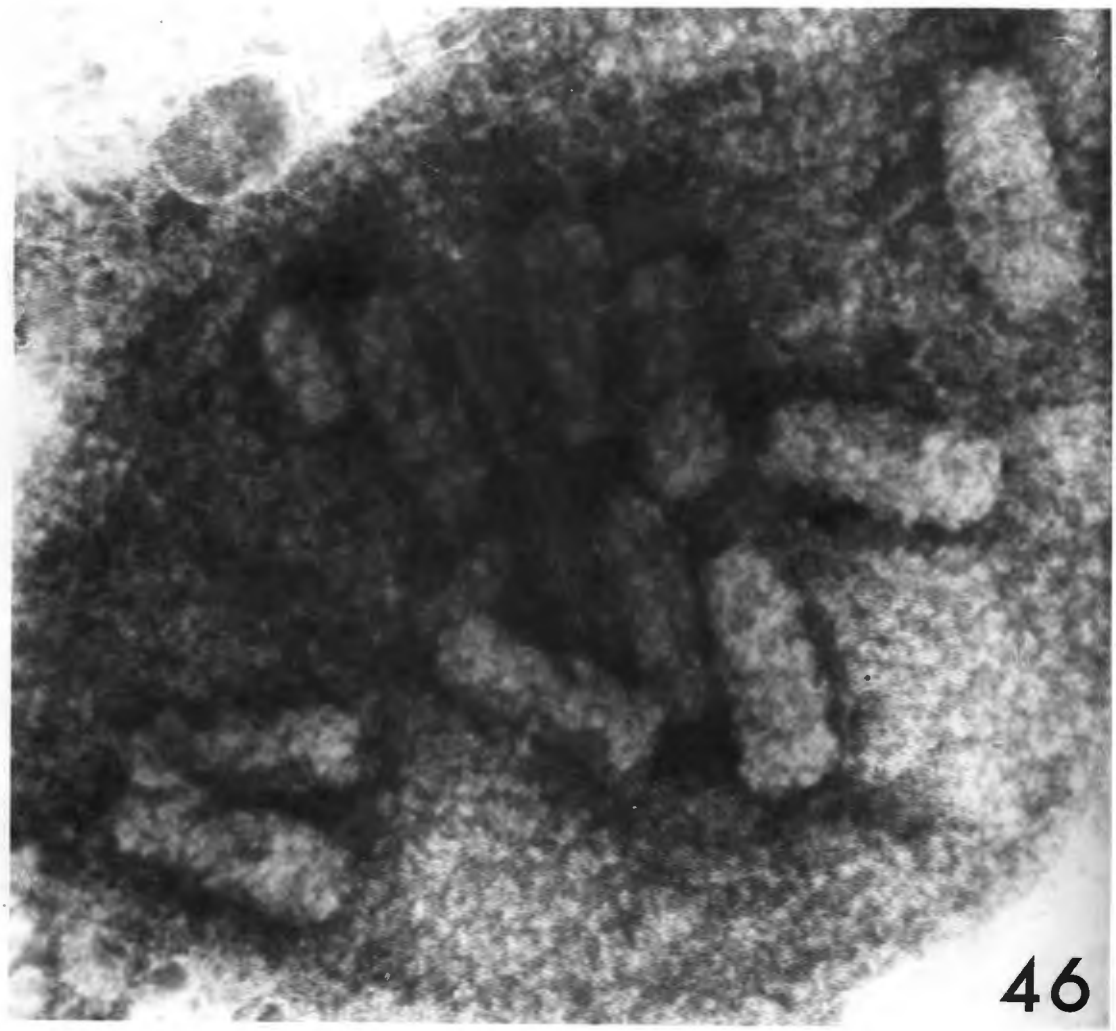


44

Fig. 45. Partially dissolved Colias electo polyhedron revealing uniform sized single virus particles. Negatively stained. Magnification X 100,000.

Fig. 46. Partially dissolved Colias electo polyhedron revealing bundles of viruses. Negatively stained. Magnification X 100,000.

45



46

Fig. 47. Free virus particles of Colias electo showing various stages of dissolution after alkali treatment. Negatively stained. Magnification X 120,000.

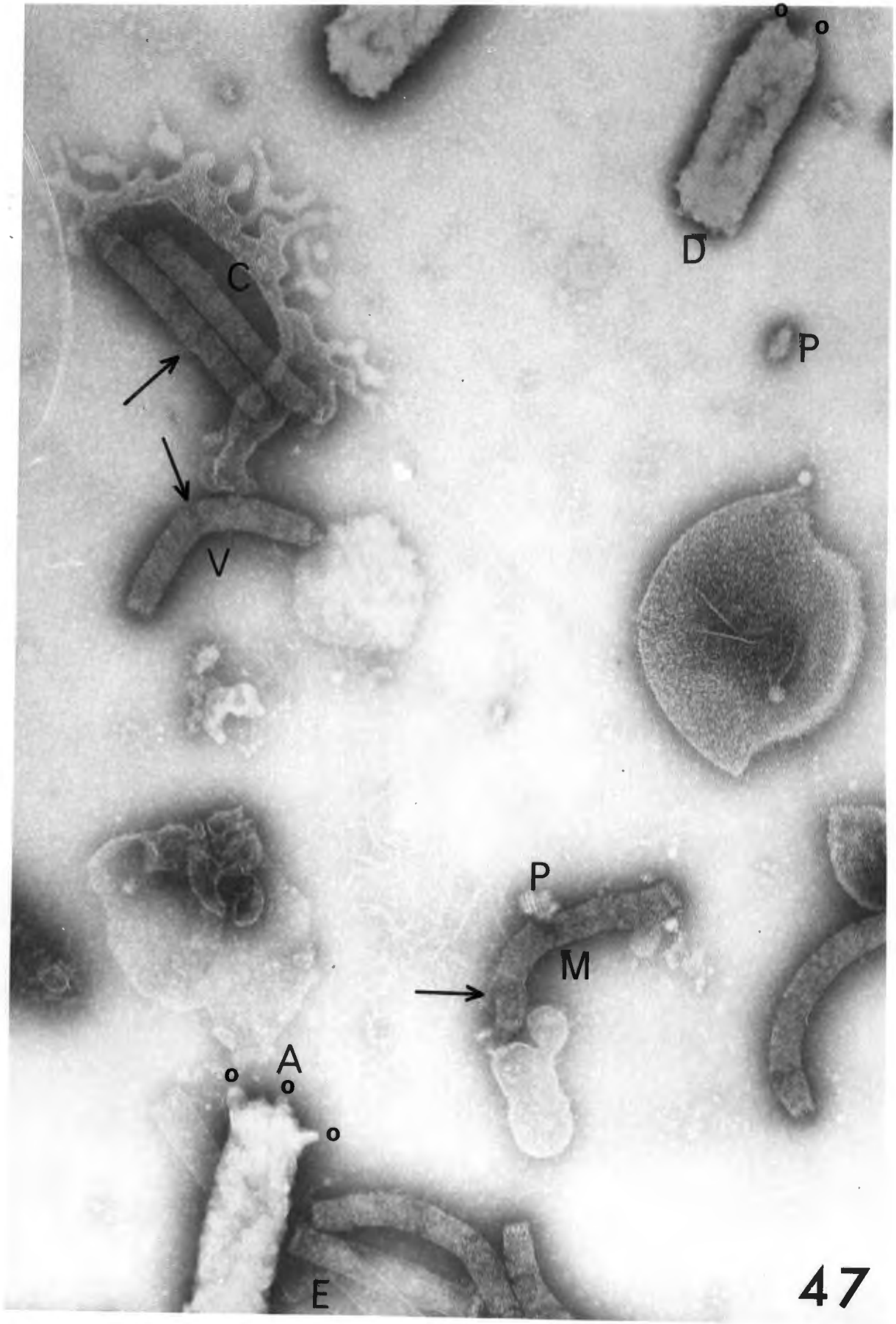
P. Fragments of polyhedral protein showing the lattice.

A and D. Bundles of virus particles within the outer membrane. Note the projections at the ends (o).

C. Two virus particles shedding their common outer membrane.

M and V. Virus particles within the inner membrane. Note the different structures at the two ends of each virus particle and the transverse lines indicated by arrows.

E. Empty inner membranes. Note the capsomeres and the structures at both ends.



47

Figs. 48-50. Virus particles of *Colias electo* within the outer membrane showing projections or protrusions. Negatively stained.

Fig. 48. S. A single virus particle showing one projection.

B. A bundle showing two projections.

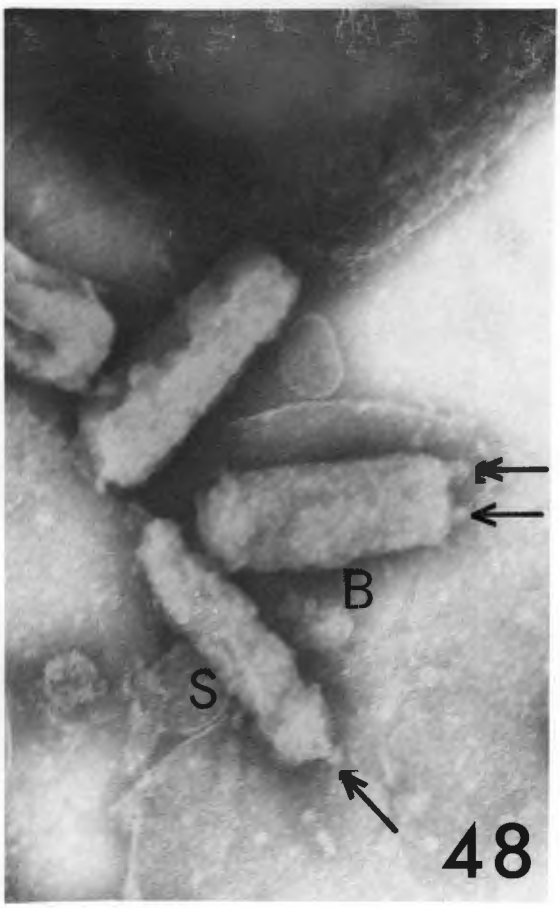
Arrows indicate projections.
Magnification X 120,000.

Fig. 49. A single virus particle showing a loop-like protrusion at one end (arrow).
Magnification X 120,000.

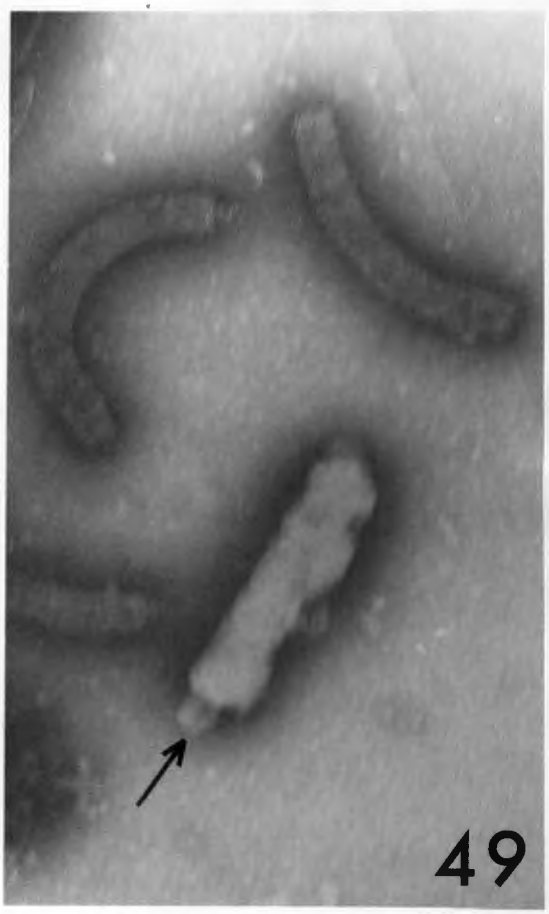
Fig. 50. D. A bundle of virus particles within the outer membrane showing four projections at each end (arrows). The projection at lower right is a protrusion of the nucleoprotein.

M. A virus particle within the inner membrane showing typical structures at both ends and the transverse lines.

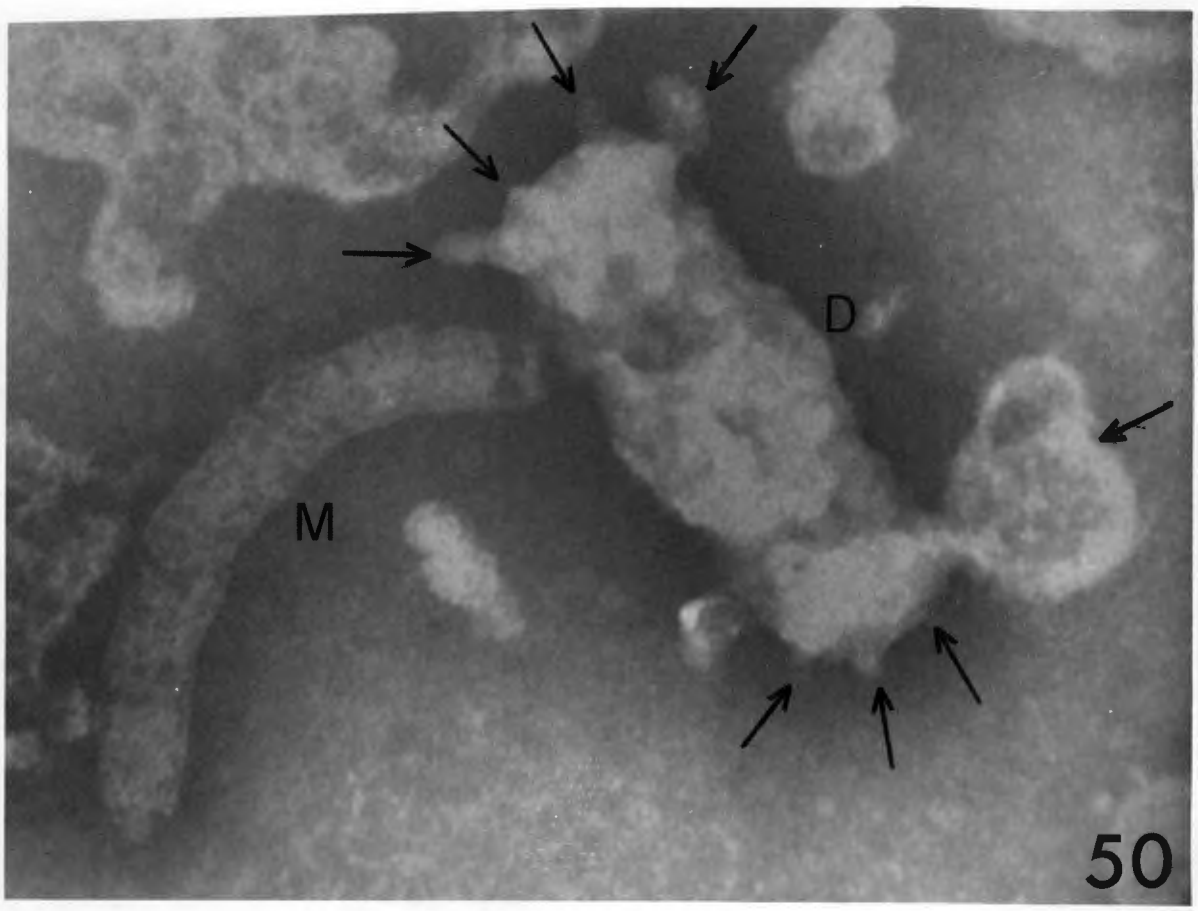
Magnification X 240,000.



48



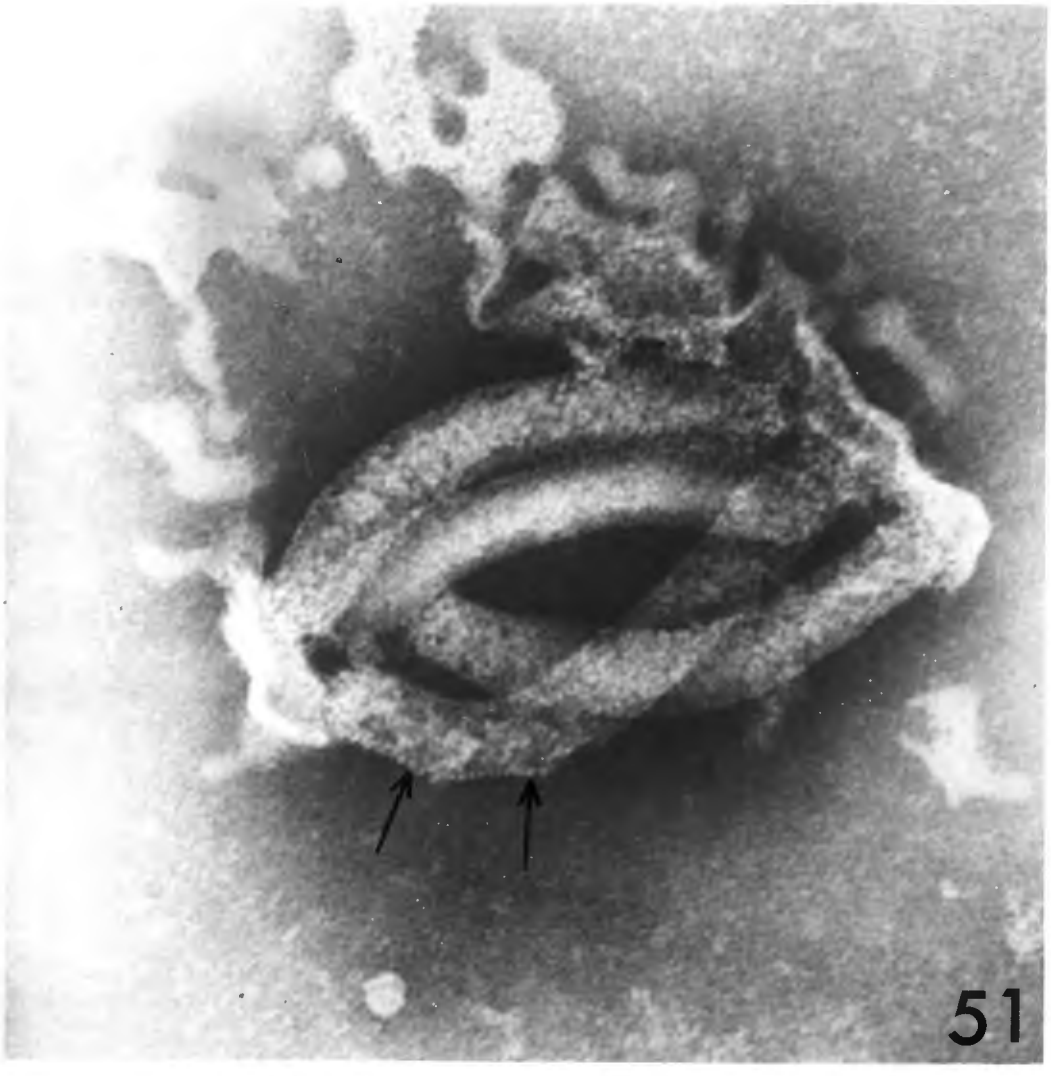
49



50

Fig. 51. A bundle of four virus particles of Colias electo shedding the common outer membrane. The latter is attached to the two ends of all four particles. Arrows indicate capsomeres. Negatively stained. Magnification X 240,000.

Fig. 52. A virus particle of Colias electo within the inner membrane which has ruptured at one end. The nucleoprotein helix is clearly noticeable inside the virus particle. At the disrupted end (arrow), two uncoiled bands are seen. Negatively stained. Magnification X 120,000.


51

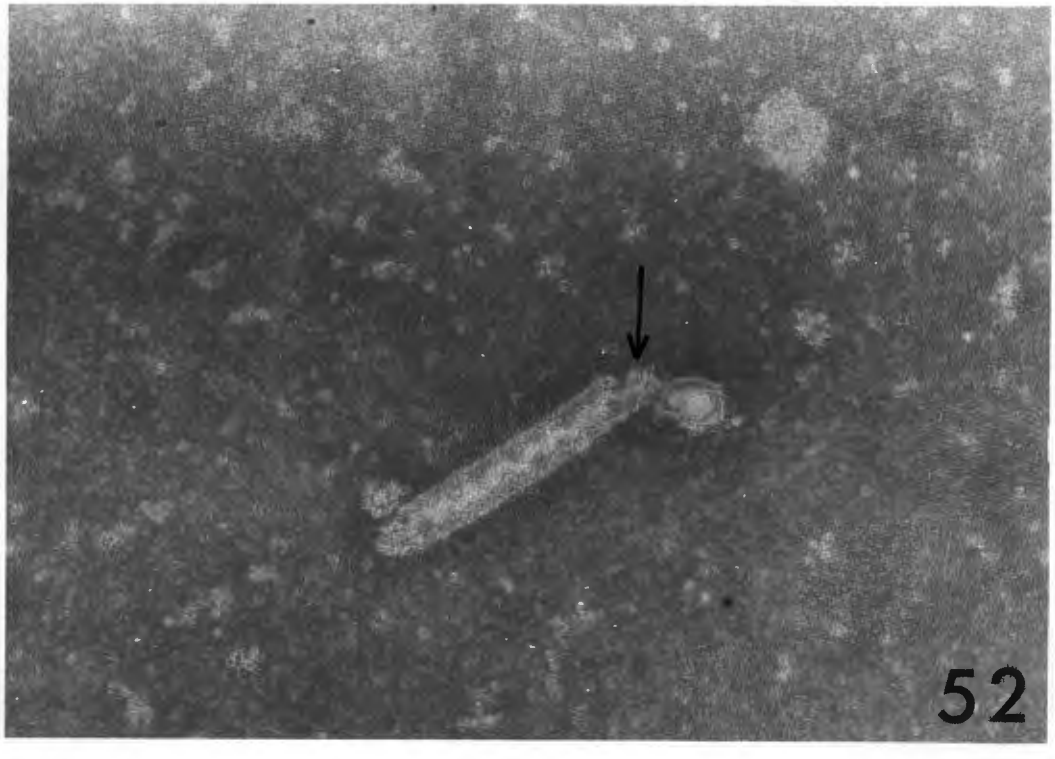
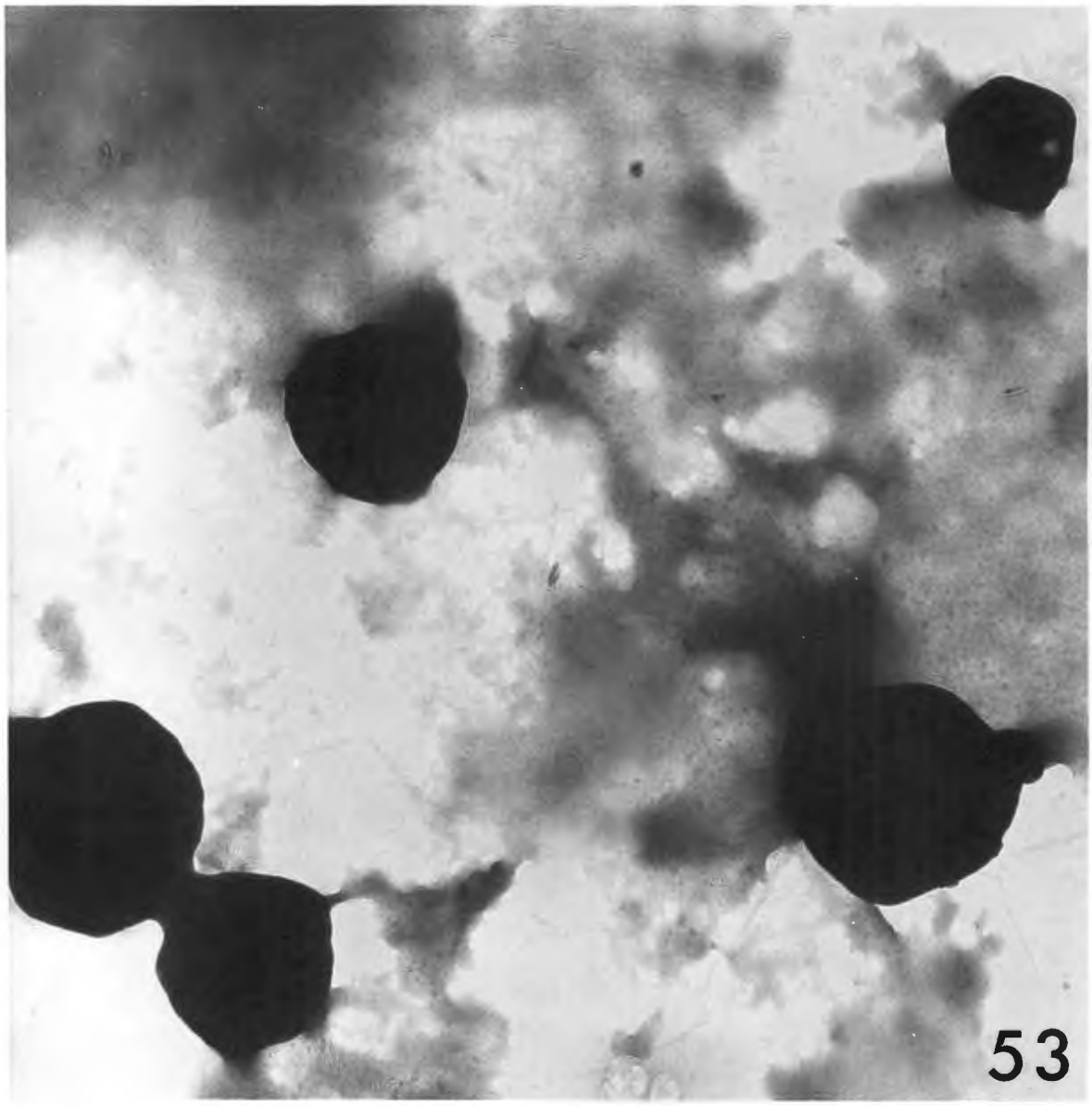
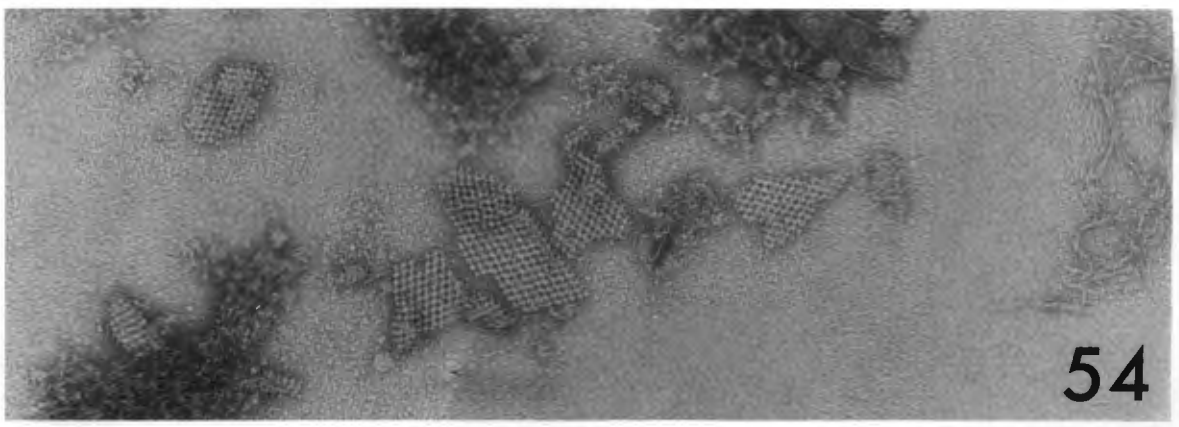

52

Fig. 53. Irregular hexagonal Heliothis zea polyhedra of various sizes. Negatively stained. Magnification X 25,000.

Fig. 54. Fragments of Heliothis zea polyhedral protein revealing the regular cubic paracrystalline arrangement of the lattice. Negatively stained. Magnification X 120,000.



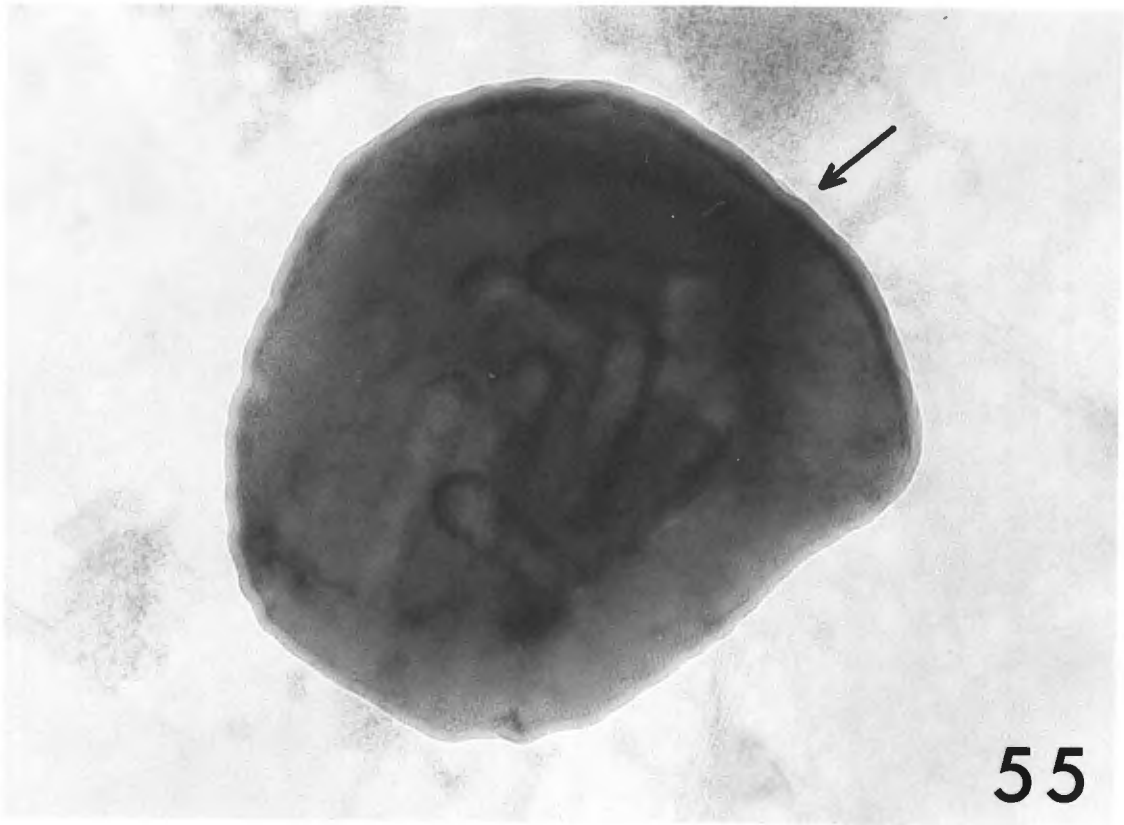
53



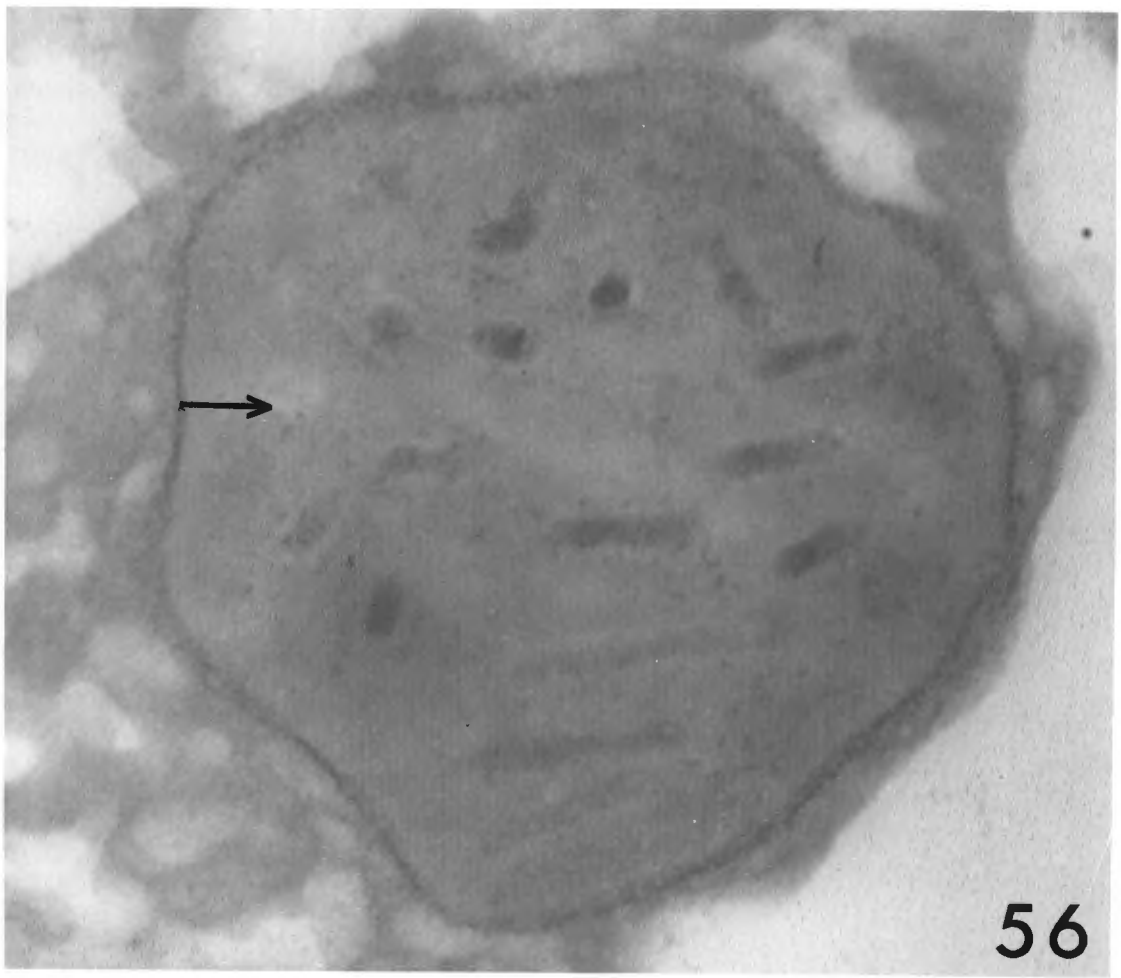
54

Fig. 55. A polyhedron of Heliothis zea revealing the surrounding membrane (arrow), the lattice arrangement of the polyhedral protein and the occluded rod-shaped virus particles. Negatively stained. Magnification X 120,000.

Fig. 56. Thin section of Heliothis zea polyhedra. The single virus particles are surrounded by the outer membrane and are randomly distributed. Note the deeper staining of the polyhedral membrane, the 'dense bands' pattern of the protein lattice and the different appearance of a virus particle sectioned through the outer membrane (arrow) compared with the others, sectioned through both the outer and inner membrane. Magnification X 120,000.



55



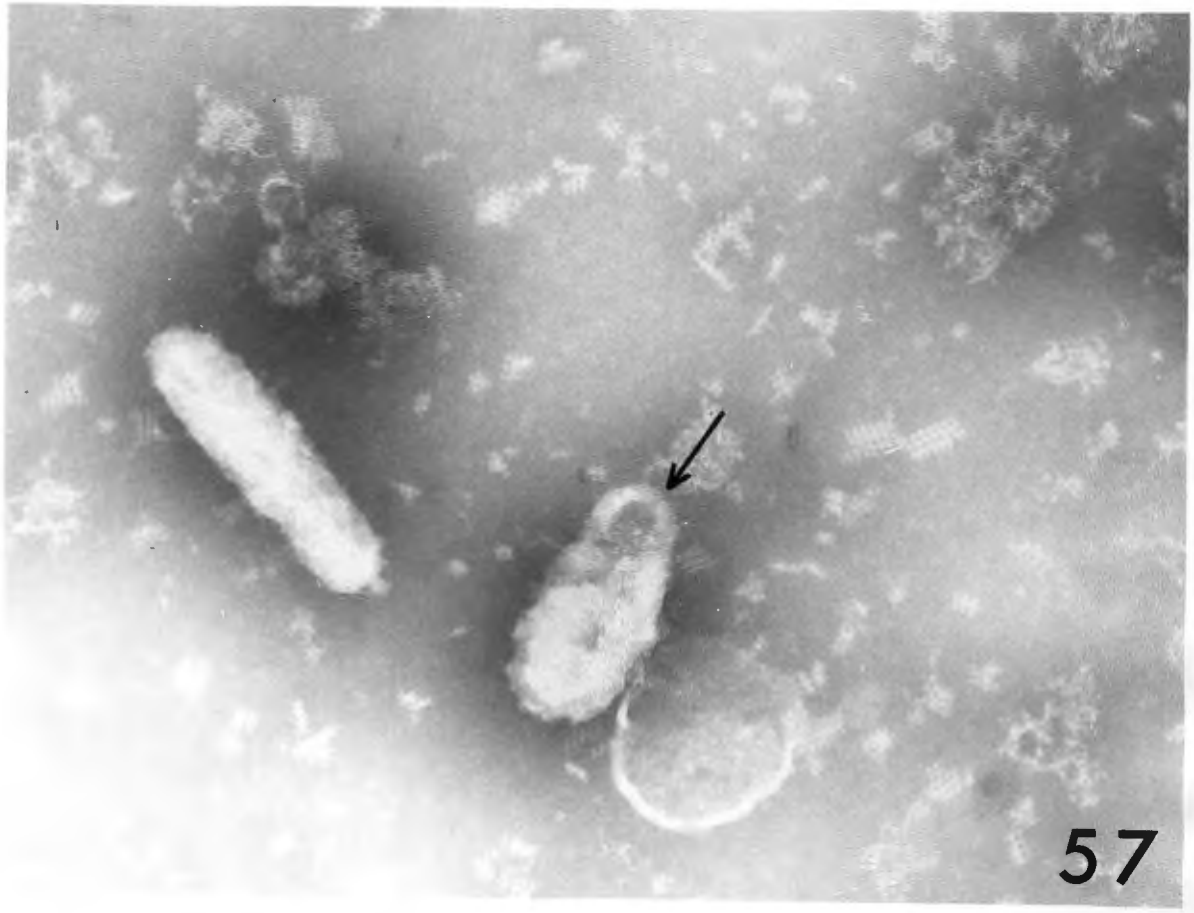
56

Figs. 57-59. Virus particles of *Heliothis zea* within the outer membrane. Negatively stained. Magnification X 120,000.

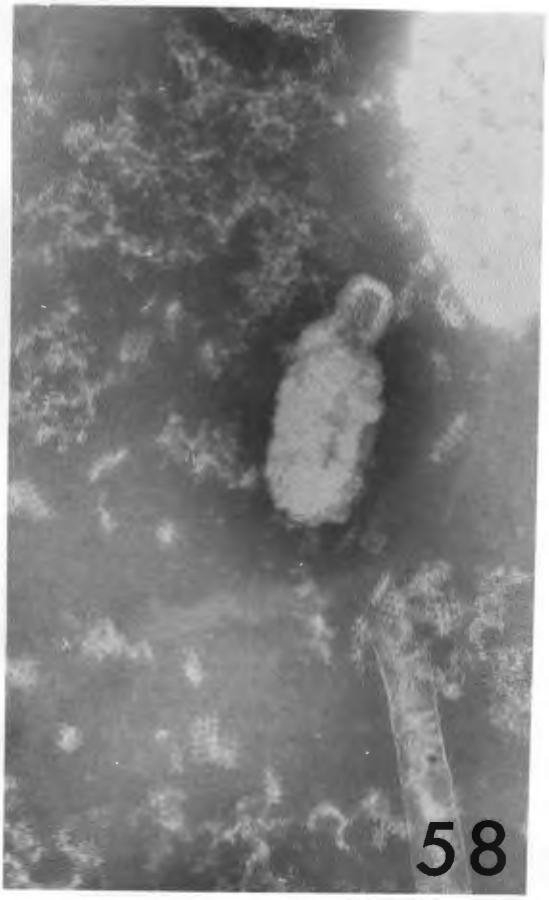
Fig. 57. A typical straight rod-shaped virus is seen on the left. The virus particle on the right is bent like a horseshoe and has projections at both ends that touch each other (arrow) to form a loop-like structure.

Fig. 58. A virus particle bent like a horseshoe, showing a projection of a loop at one end only.

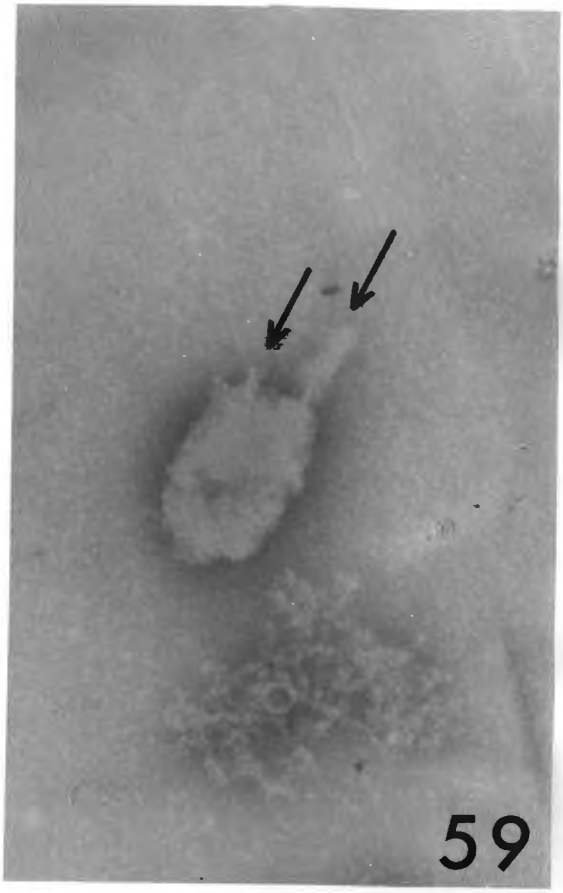
Fig. 59. A virus particle bent like a horseshoe, showing straight projections at both ends (arrows).



57



58



59

Fig. 60. Virus particles of Heliothis zea within the inner membrane emerging from the double outer membrane. Negatively stained. Magnification X 120,000.

A. The outer membrane after breaking in the centre to form two spheres.

B. Spheres of ruptured outer membrane.

C. The outer membrane broken at one end. Note the helical nucleoprotein.

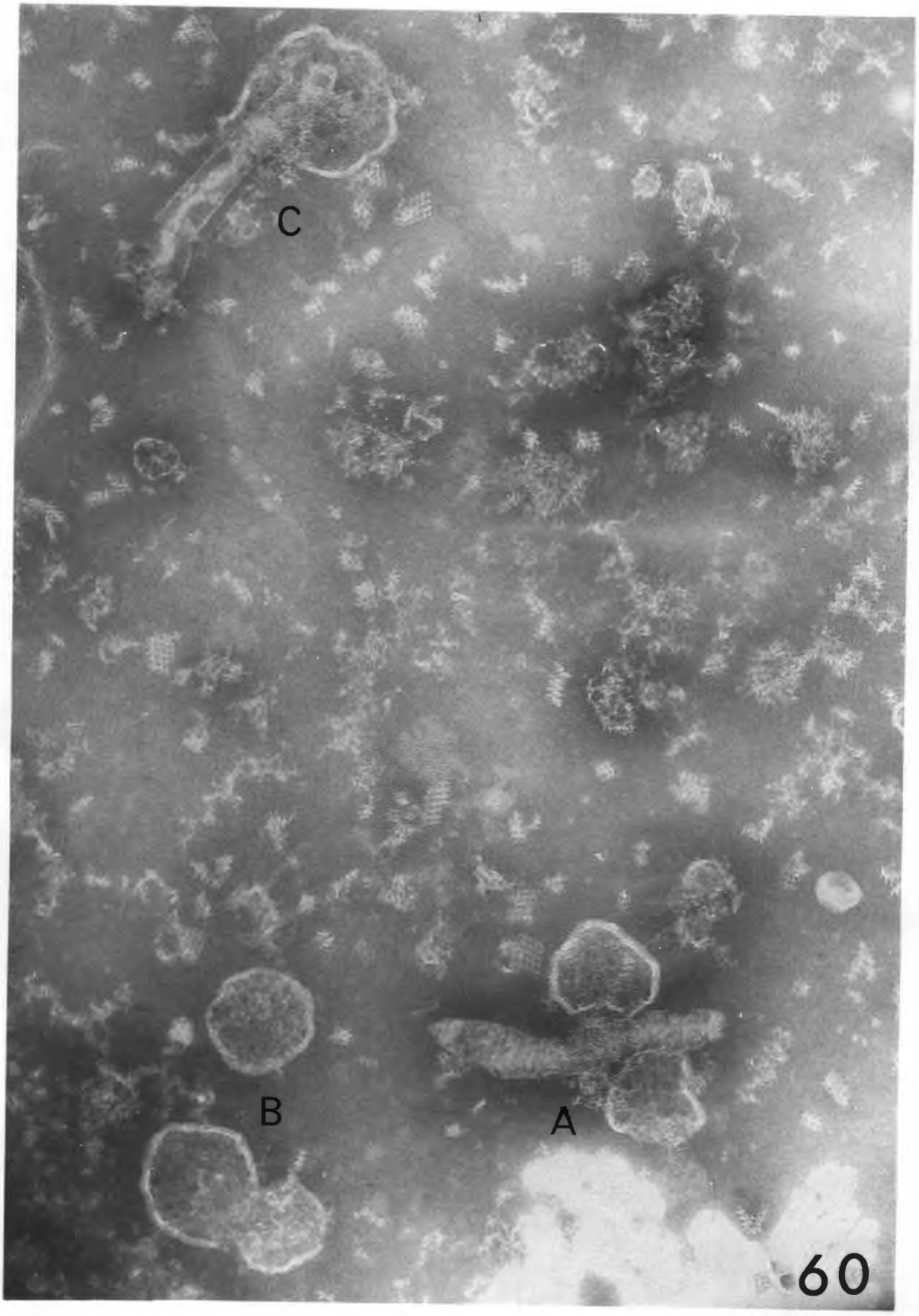

C
B
A
60

Fig. 61. Free virus particles of Heliothis zea within the inner membrane in a twisted and compressed form. Negatively stained. Magnification X 120,000.


61

Figs. 62-63. Empty inner membranes of the virus of Heliothis zea revealing the capsomeres along the sides. Negatively stained. Magnification X 120,000.

Fig. 62. T. Typical tube shape of the inner membrane.

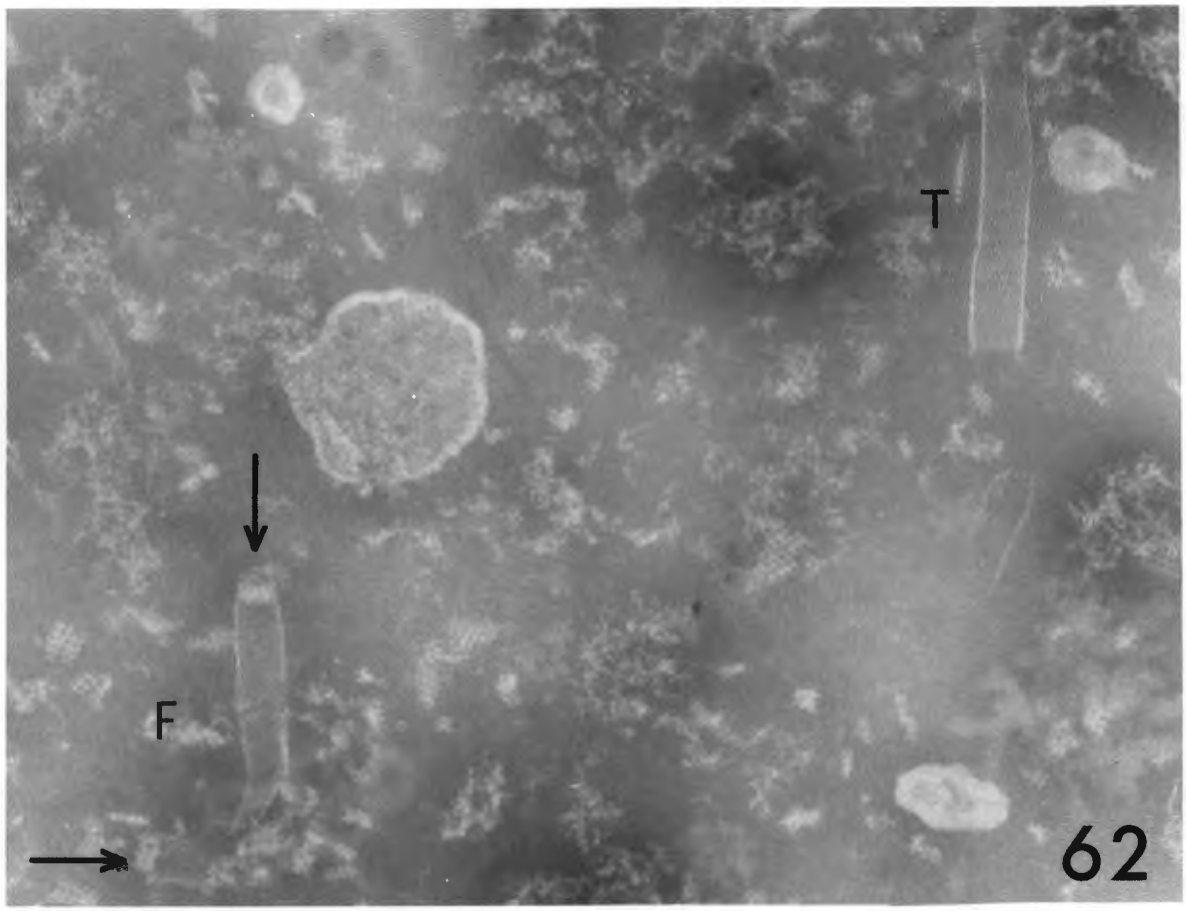
F. Folded empty inner membrane with claw-setting at both ends (arrows).

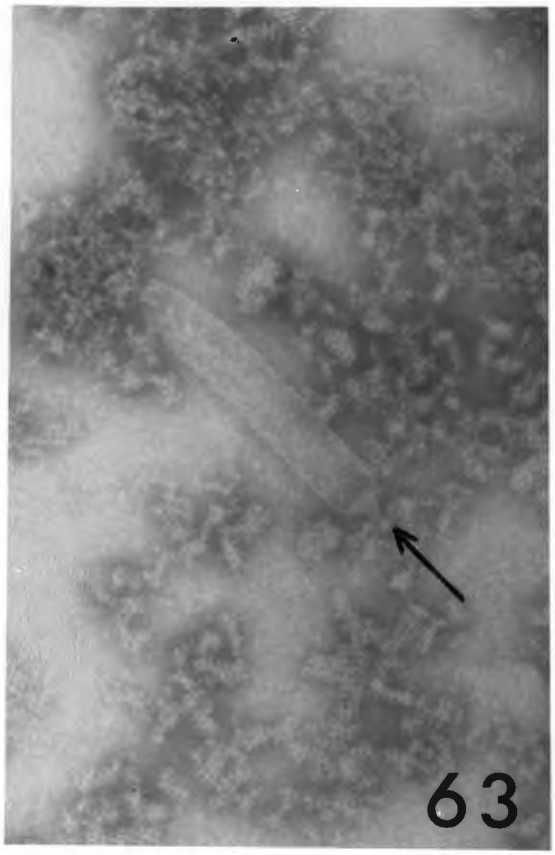
Fig. 63. The nipple and the claw-setting at one end (arrow) of the empty inner membrane.

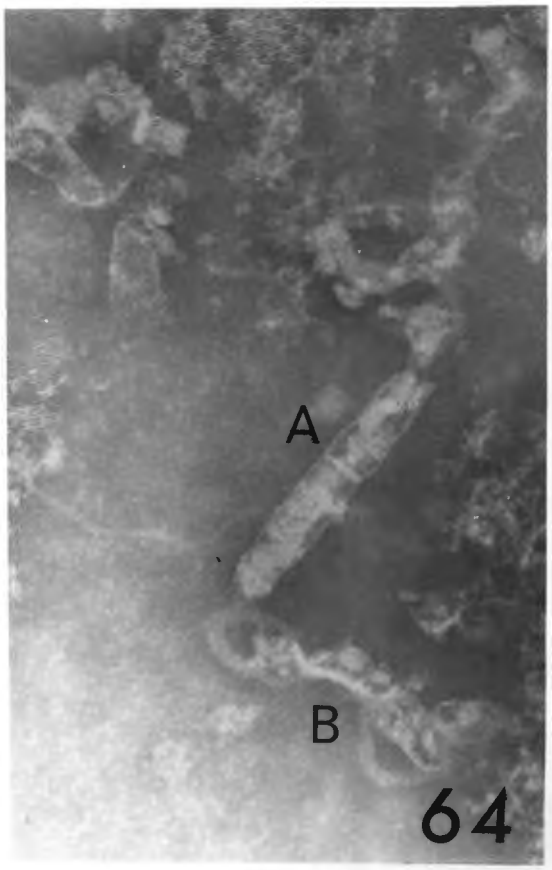
Fig. 64. The nucleoprotein of the virus of Heliothis zea. Negatively stained. Magnification X 120,000.

A. The coiled, transversely striated helix of the nucleoprotein inside the inner membrane.

B. The freed nucleoprotein helix, partly uncoiled, forming loops about the remains of the ruptured membrane.


T
F
62


63


A
B
64

6.3.2. Characteristics of the granulosis virus of Heliothis armigera.

A. The capsules and the occluded virus particle.

The granulosis capsules, though smaller than the polyhedra, were easily observed under the light microscope. They seemed very uniform in size, with a granular appearance and were highly refractile. However, unlike the polyhedra, their exact shape and dimensions could only be ascertained by electron microscopy.

The majority of the inclusion bodies were ovocylindrical (Fig. 65). Occasionally long capsules (Figs. 69; 71), capsules shorter than usual (Fig. 68S) or square (Fig. 62H) and U-shaped capsules (Fig. 68U) were found. Cross sections through capsules showed irregular shapes (Figs. 66; 67).

The dimensions of the capsules are summarized in Table 6.3.1., ranging from 400-542 nm in length and 175-241 nm in width. The long capsules were of similar width but their length, 800-820 nm was approximately double that of average size. The cavity inside the capsule was most often of the size to contain an average rod-shaped virus particle (Figs. 65V; 68N; 72; 73; 74; 75) but was occasionally longer (Fig. 69) or unusually club-shaped (Figs. 70; 71).

The capsules consisted of a broad protein mass occupying approximately twice the width of the virus particle and were surrounded by a thick double membrane (Fig. 65). After partial digestion the lattice pattern of the capsular protein was exposed (Figs. 65V; 74). The cavity inside the capsule containing the virus particle (Fig. 65V) or the cavity in the empty capsule (Figs. 73; 74; 75) was clearly demonstrated

by the penetrating stain. The virus inside the capsule was generally rod-shaped and straight but sometimes slightly curved (Fig. 68U). After release from the capsule, while still within the outer membrane, it was also generally straight (Fig. 76) although occasionally curved (Fig. 74).

The release of virus particles after alkali treatment may be achieved in various ways. Commonly there was a longitudinal split of the capsule (Fig. 72) resulting in the capsule separating into two halves (Fig. 73). A cross break was also common giving rise to various sized U-shaped open capsules (Fig. 68U). The escape of the virus through a breach at one end (Fig. 75) or in the side wall (Fig. 74) was also observed.

B. The virus particle within the outer membrane.

The virus particle within its outer membrane was opaque (Figs. 67; 74; 76). Outside the capsule the size seemed more uniform than when it was twisted and compressed while still occluded by the capsule (Table 6.3.1.). In thin sections the width of the virus within the outer and inner membranes was less than in negatively stained suspensions (Table 6.3.1.). There was a space between the outer membrane and the occluded virus (Figs. 66; 78). Only once was a projection or protrusion found on one end of the virus (Fig. 76 arrow). When the virus was released from the outer membrane the latter curled into one or more spheres situated at some point on the long axis of the virus, exposing the double structure of the membrane (Figs. 75S; 79). The dimensions of the virus particles within the outer membrane were fairly constant (Table 6.3.1.).

C. The virus particles within the inner membrane.

The virus particle within the inner membrane was much more opaque (Figs. 79; 80M) than the nuclear-polyhedrosis virus particles described earlier. The claw-setting could be seen faintly on both ends of full membranes (Figs. 79; 80M, R) and of empty inner membranes (Fig. 81); a nipple was seen at one end (Fig. 79 arrow). Occasionally the alkali penetrated the particle leaving only the two ends and the membranes intact as shown by the areas of opacity (Fig. 78). The virus particle was sometimes seen to be fractured transversely and folded over while still inside the partially dissolved outer membrane as in Fig. 77. The same occurred occasionally when the particle was free (Fig. 80R) and, in this instance, the uncoiling nucleoprotein was seen escaping from the inner membrane. The inner membrane sometimes ruptured at one end as in Fig. 81; this picture also portrays the fine structure of the inner membrane showing its capsomeres. The virus particles within the inner membrane appeared narrower and longer than those within the outer membrane, indicating the compression within the outer membrane. Their dimensions were fairly constant (Table 6.3.1.) except some rare long virus particles, approximately double the average size (Fig. 82).

6.3.3. Virus-like structures.

In many preparations of the nuclear-polyhedrosis viruses of Heliothis zea and Colias electo, the granulosis virus of Heliothis armigera and the cytoplasmic-polyhedrosis virus of the silkworm, Bombyx mori, virus-like structures were seen.

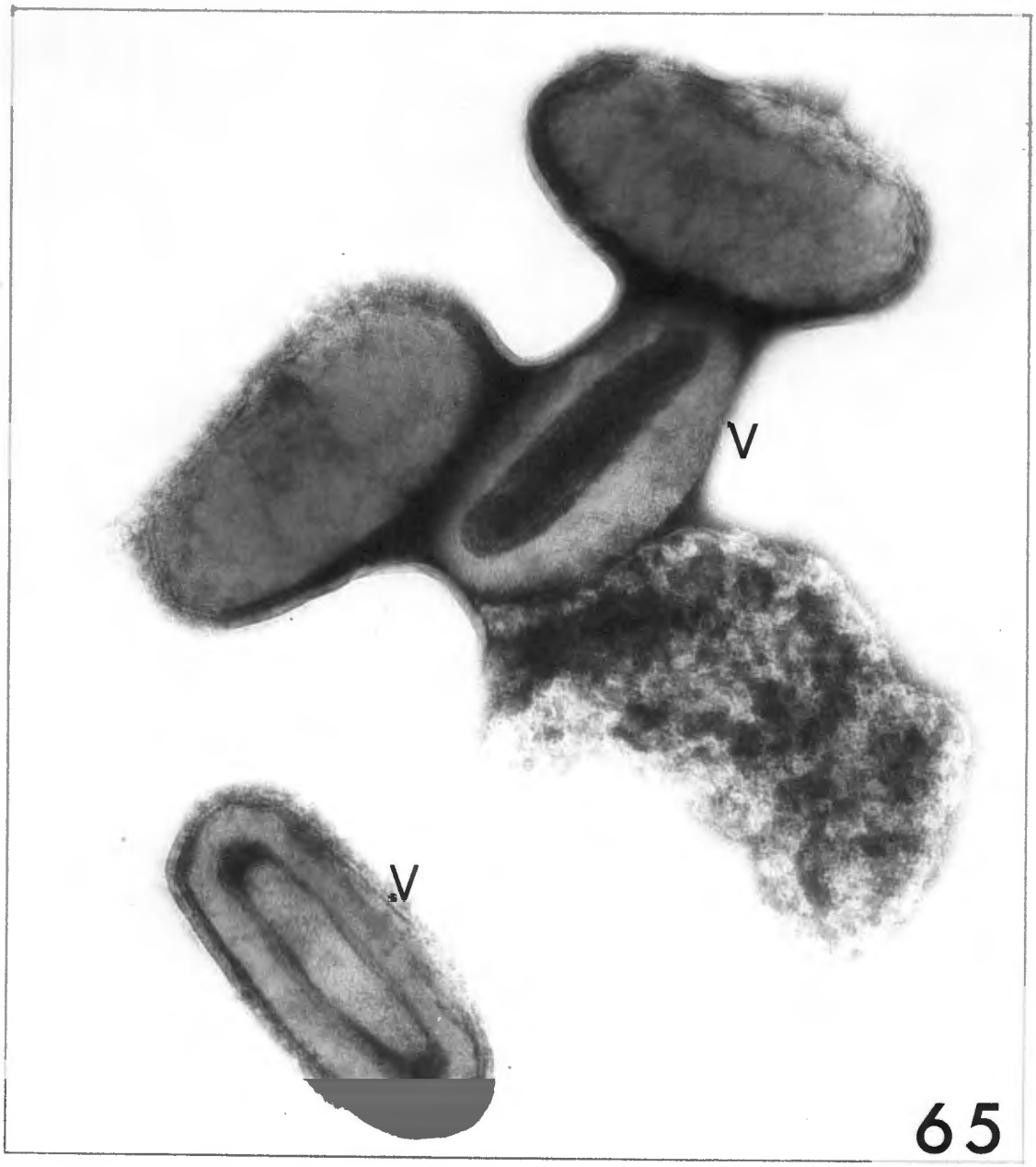
These preparations had been treated with alkali, ultrasound or caesium chloride.

The structure appeared in two distinct forms. The one was thin and showed one central band (Fig. 83A) and the other showed two bands with a central core (Fig. 83D). They varied in width from 40 to 54 nm for the latter structure and approximately 20 nm for the former. The variations in length were greater, from 117 to 1714 nm, each particle surrounded by a membrane-like structure. Shorter broken pieces were seen. Aggregation was noticed along the side-walls but never end to end.

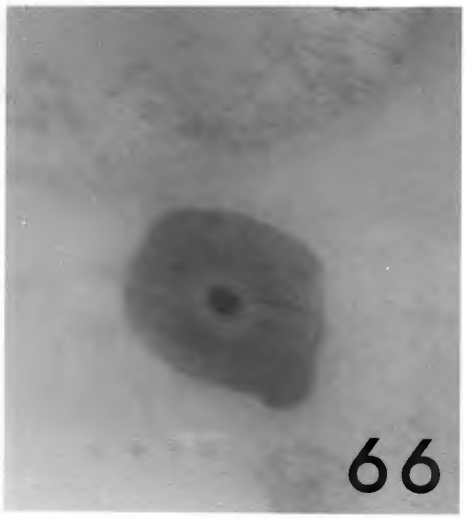
Fig. 65. Intact capsules of Heliothis armigera. Two of them (V) have been penetrated by the stain and show the single occluded virus particle. The double membrane around each capsule is noticeable and the lattice arrangement of the capsule protein is also seen on V. Negatively stained. Magnification X 120,000.

Fig. 66. Thin cross section through a capsule of Heliothis armigera showing the virus particle and the surrounding outer membrane. Magnification X 120,000.

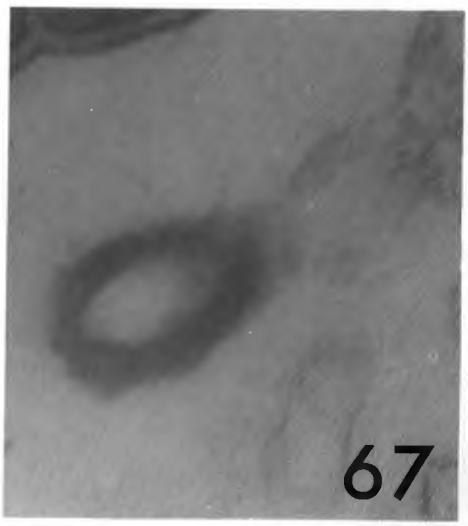
Fig. 67. An oblique section through a capsule of Heliothis armigera. The outer membrane was sectioned through and the occluded virus is not seen. Magnification X 120,000.



65



66



67

Fig. 68. Capsules of Heliothis armigera of unusual shape. Negatively stained. Magnification X 120,000.

H. A square capsule with a hollow spherical core.

S. A capsule shorter than the average showing sharp corners and a dumb-bell-shaped cavity from which the virus particle has escaped.

U. A U-shaped capsule with one end broken open. The capsule cavity is slightly curved.

N and A. Usual capsules.

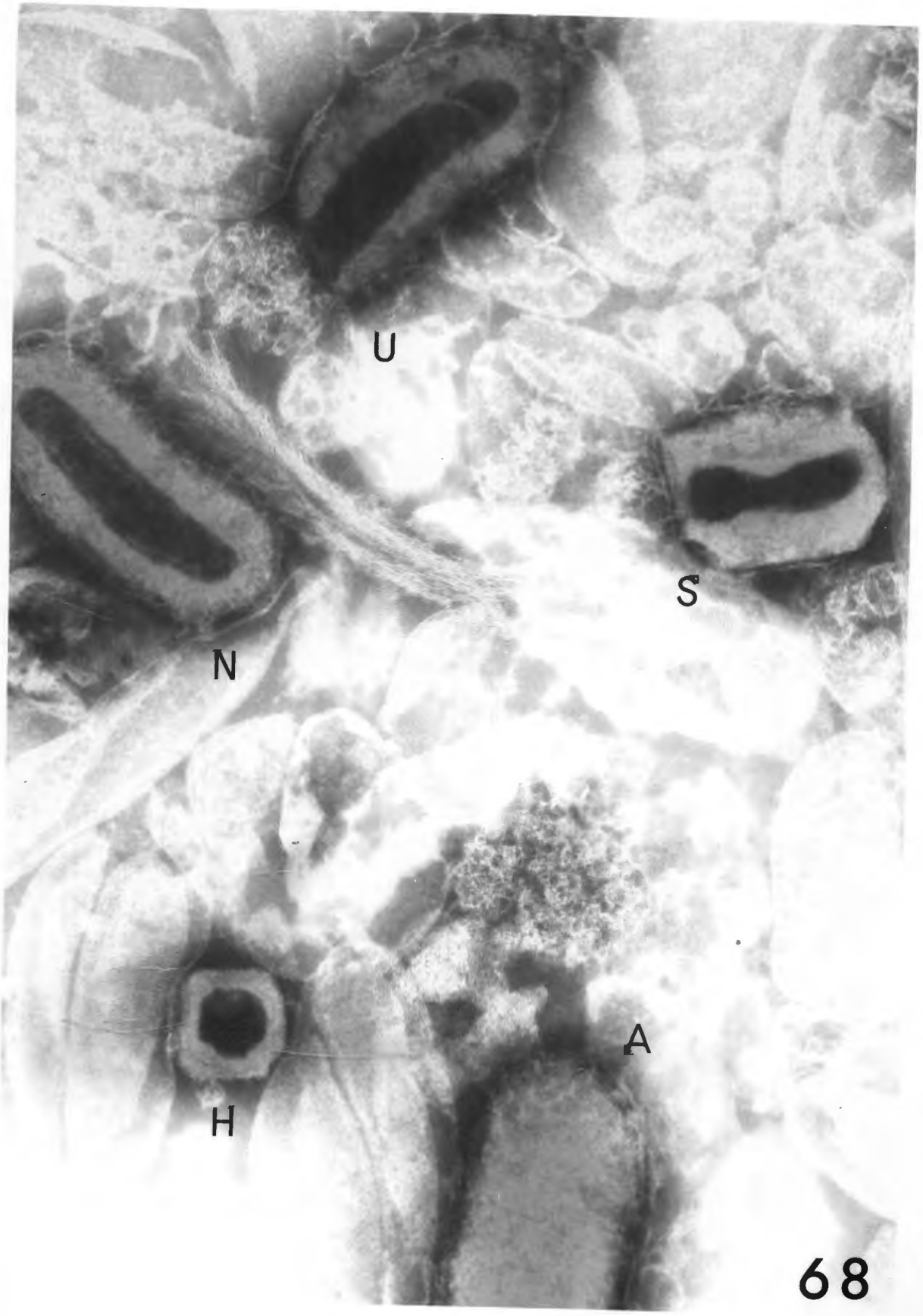
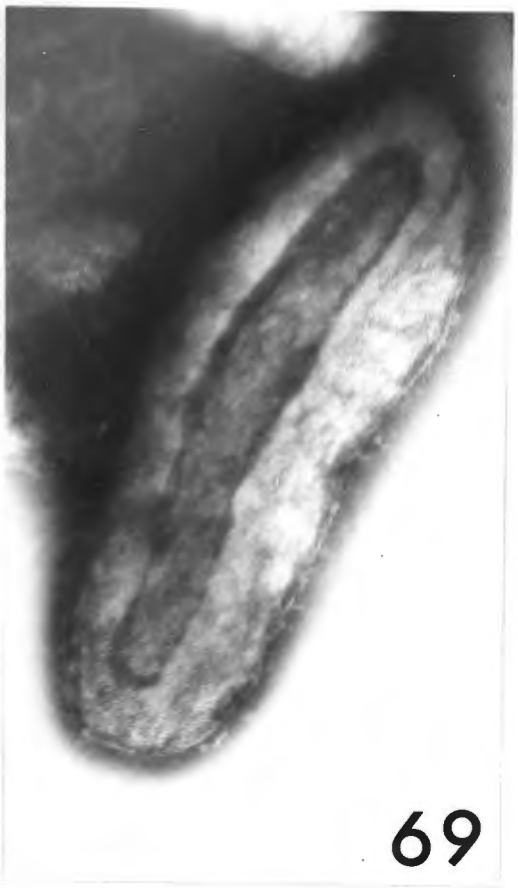

U
S
N
A
H

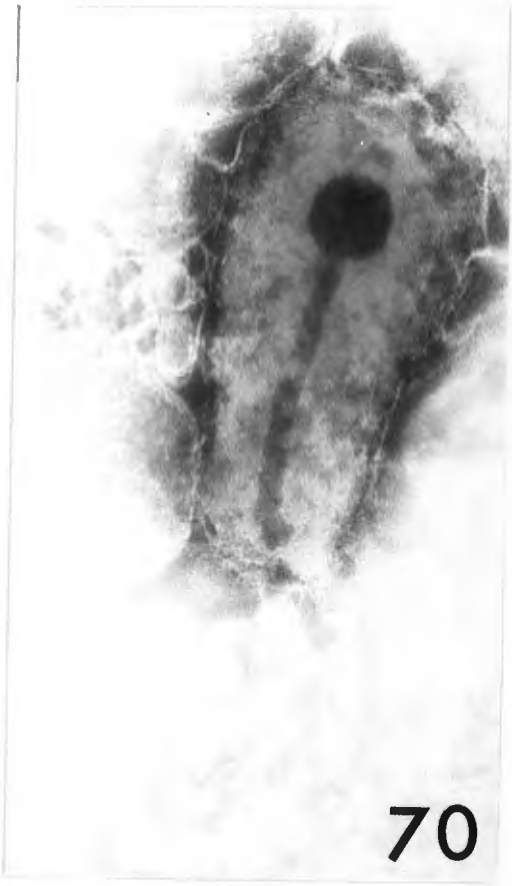
Fig. 69. An unusually long capsule of Heliothis armigera with a double length rod-shaped occluded virus. Negatively stained. Magnification X 120,000.

Fig. 70. A capsule of Heliothis armigera showing an unusual club-shaped cavity. Negatively stained. Magnification X 120,000.

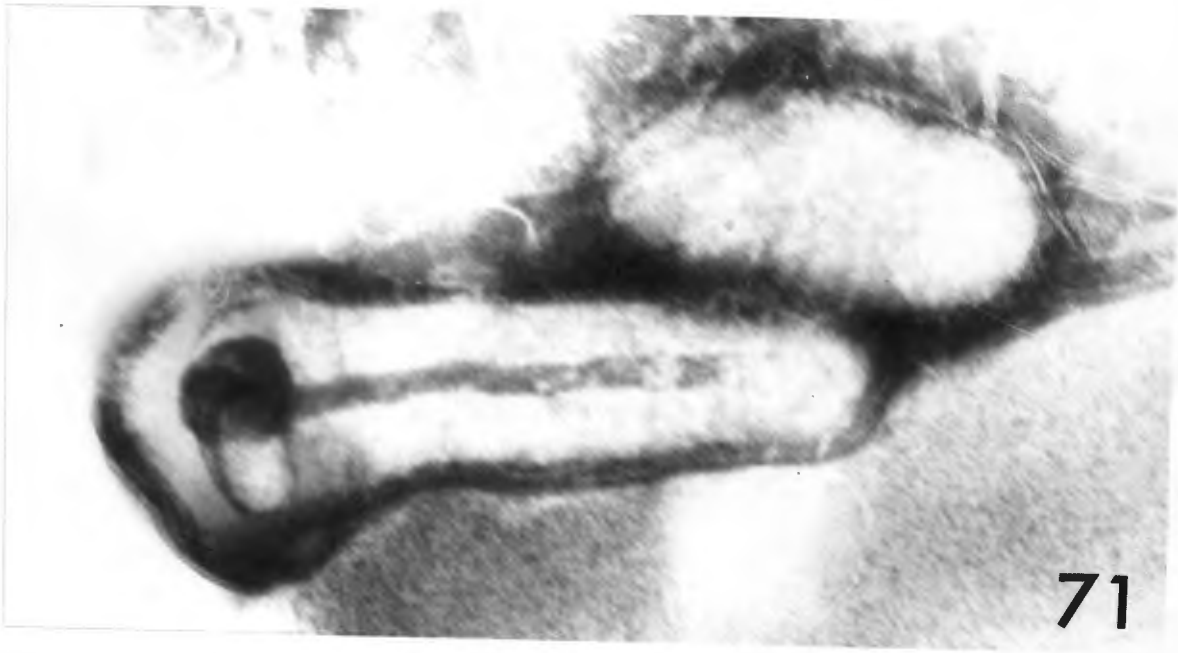
Fig. 71. An unusually long capsule of Heliothis armigera below, compared with one of normal size above. The unusual capsule cavity resembles that seen in Fig. 70. Negatively stained. Magnification X 120,000.



69



70



71

Figs. 72-75. Different breaks in the Heliothis armigera capsule showing release of the virus particle. Negatively stained. Magnification X 100,000.

Fig. 72. A wheat-like capsule with a longitudinal split.

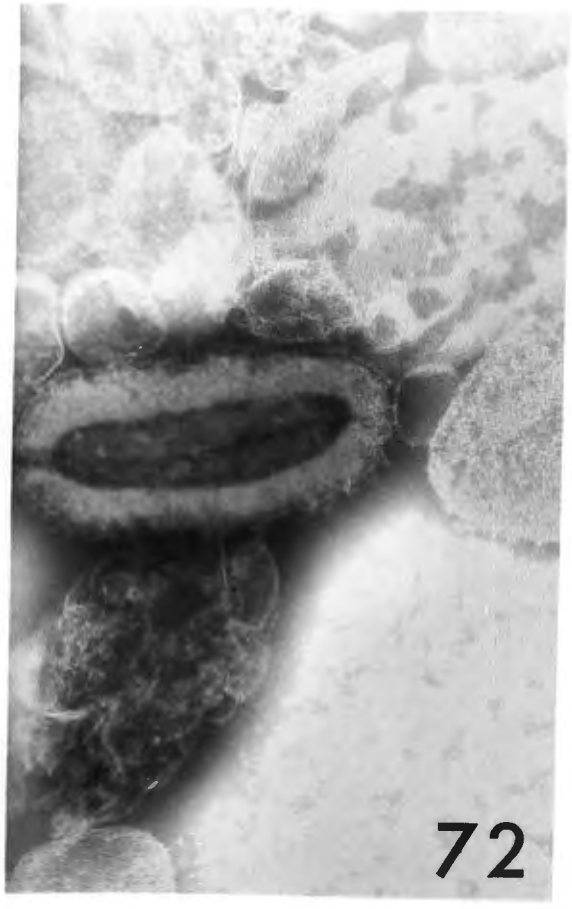
Fig. 73. A capsule broken at both ends.

Fig. 74. A virus particle within the outer membrane emerging from a capsule through an aperture in the side wall. The lattice arrangement of the capsular protein is clearly seen.

Fig. 75. A capsule broken at one end.

S. The double outer membrane of the released virus particle in a form of two spheres.

N. The virus particle within a partially dissolved inner membrane exposing the nucleoprotein.


72


73


74

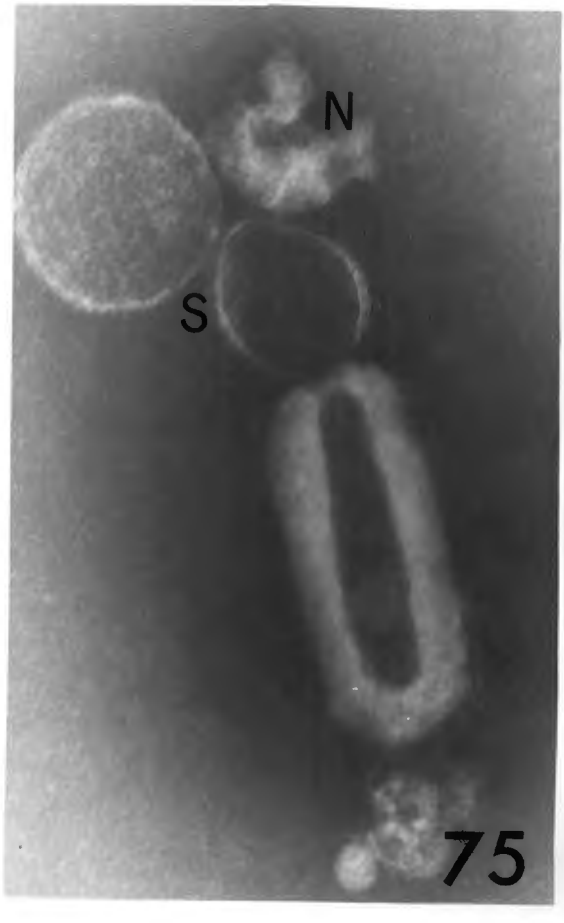
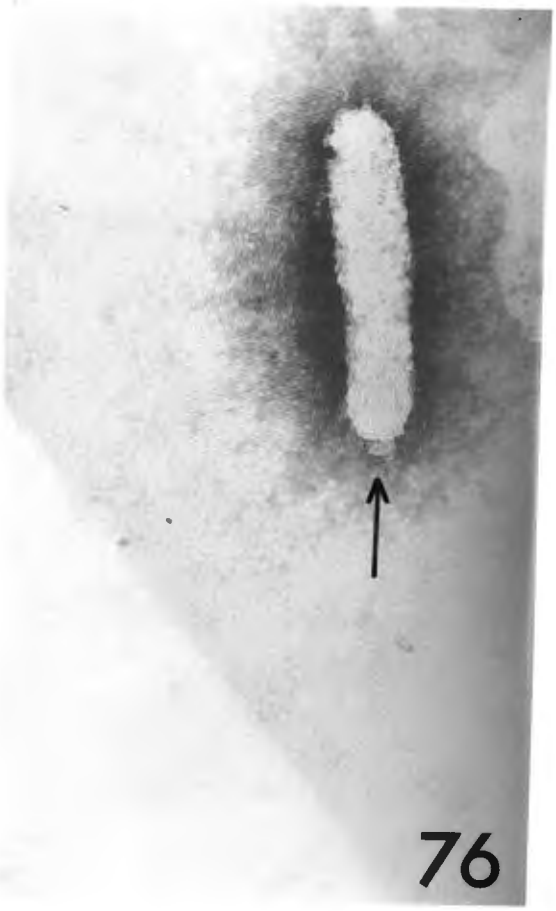

N
S
75

Fig. 76. A virus particle of Heliothis armigera within the outer membrane showing a projection at one end (arrow). Negatively stained. Magnification X 120,000.

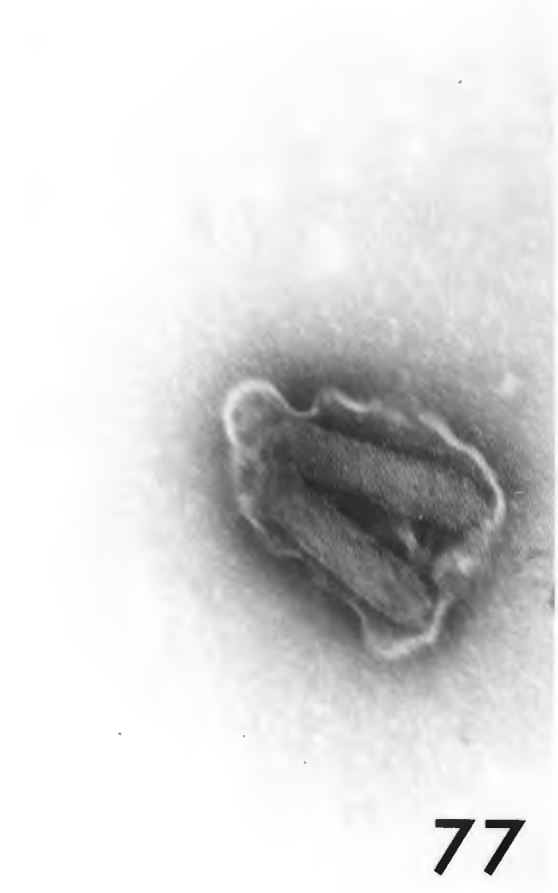
Fig. 77. A bent and fractured virus particle of Heliothis armigera within its partially dissolved outer membrane. Negatively stained. Magnification X 120,000.

Fig. 78. A virus particle of Heliothis armigera within the outer membrane. The central part has been digested while the two membranes and the ends are intact. The outer membrane is thinner at the ends than in the centre. Negatively stained. Magnification X 120,000.

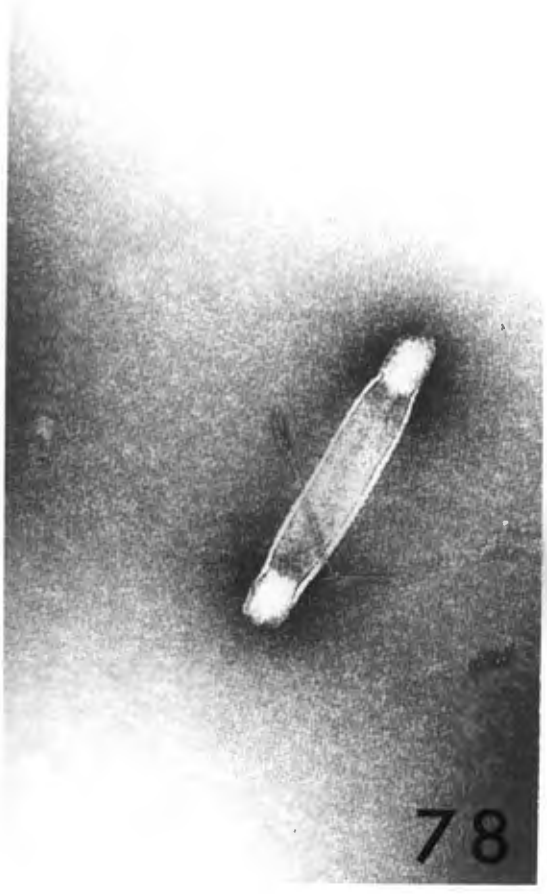
Fig. 79. A virus particle of Heliothis armigera within its inner membrane, shedding the outer membrane in the form of two spheres. Claw-setting and a nipple are seen at one end (arrow). Negatively stained. Magnification X 120,000.



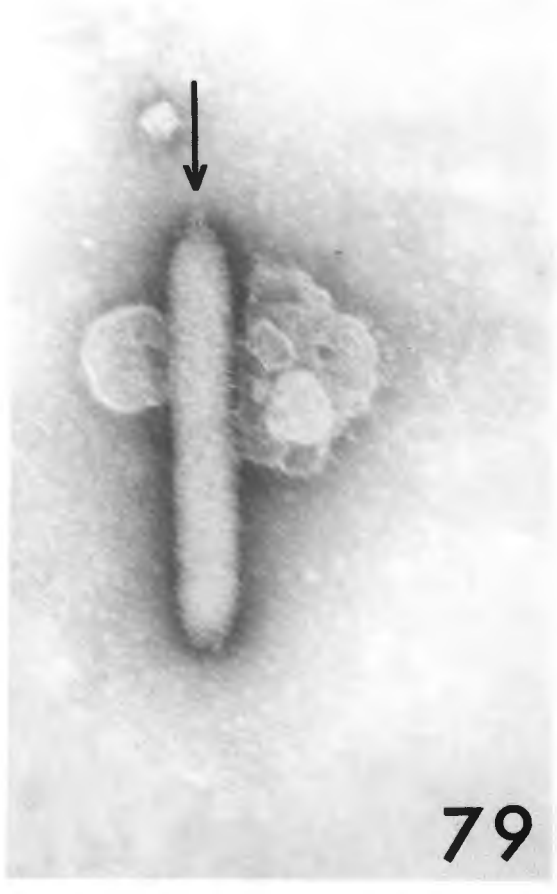
76



77



78



79

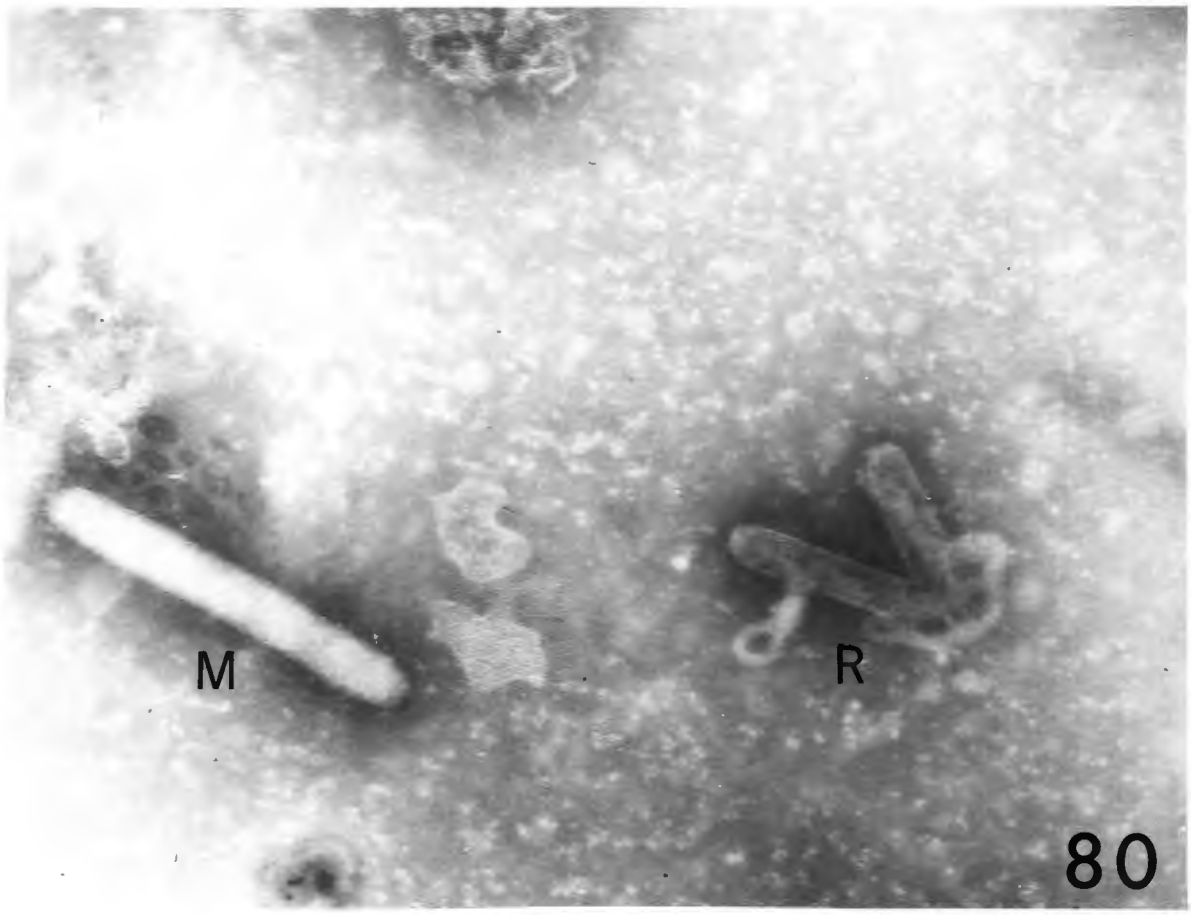
Fig. 80. Virus particles of Heliothis armigera within the inner membrane. Negatively stained. Magnification X 120,000.

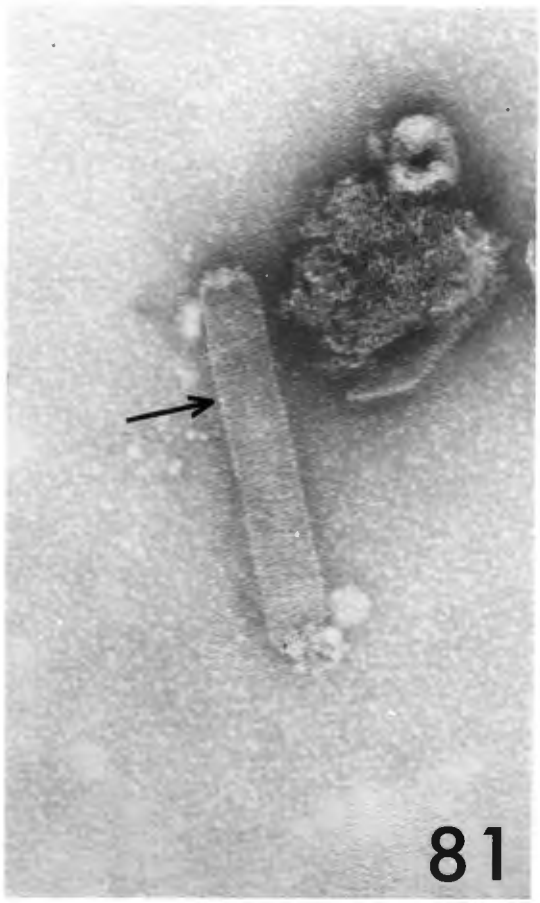
M. Intact particle with the claw-setting at both ends and the nipple at top end.

R. Ruptured particle releasing the nucleoprotein.

Fig. 81. An empty tube-like inner membrane of Heliothis armigera virus showing the claw-setting at top end. The capsomeres (arrow) of the inner membrane are visible. Negatively stained. Magnification X 120,000.

Fig. 82. An unusually long virus rod of Heliothis armigera. Negatively stained. Magnification X 120,000.


M
R
80


81

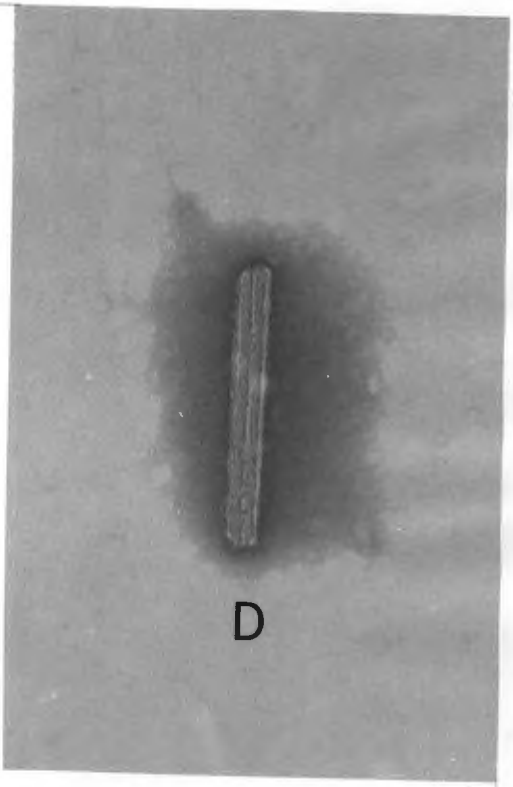

82

Fig. 83. Virus-like structures found in most preparations. Negatively stained. Magnification X 120,000.

A. A thin structure showing one central band.

D. Structures of two different lengths showing two bands with a central core.


D


D

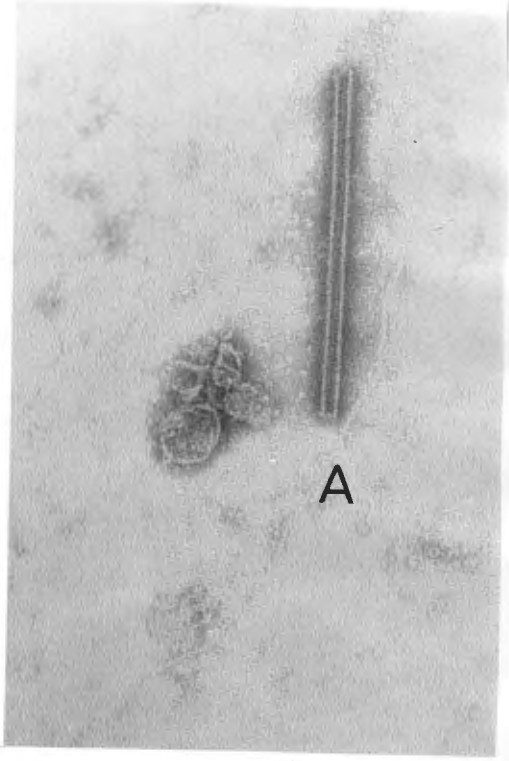

A

Table 6.3.1. Measurements of the inclusion bodies and their virus particles.

Material	Treatment (source)	Type of measurement	Polyhedrosis virus of *Colias electo*		
			Length nm	Width nm	Ratio L:W
Inclusion bodies	Suspension	Mean ± SD Range	1835 ± 616* 717 - 2800*	1632 ± 590** 642 - 2430**	
Virus in outer membrane inside the inclusion body	Suspension	Mean ± SD Range	328 ± 4 325 - 333	94 ± 30 67 - 150	4.0 2.2 - 4.9
		Mean of a single virus	325	67	4.9
		Mean of a bundle	333	125	2.7
	Thin Section	Range of a single virus	-	45 - 67	-
		Range of a bundle	-	83 - 123	-
Virus in outer membrane liberated from inclusion body	Suspension	Mean ± SD Range	313 ± 19 275 - 342	104 ± 19 58 - 125	3.1 2.3 - 5.3
		Range of a single virus	275 - 333	58 - 83	3.7 - 5.3
		Range of a bundle	292 - 342	108 - 125	2.3 - 3.0
Virus in inner membrane outside the inclusion body	Suspension	Mean ± SD Range	350 ± 30 300 - 400	47 ± 4 40 - 50	7.3 5.4 - 8.6
Virus in inner membrane inside the inclusion body	Thin Section	Range	-	25 - 33	-

Table 6.3.1. (Continued)

Material	Treatment (Source)	Type of measurement	Polyhedrosis virus of Heliothis zea		
			Length nm	Width nm	Ratio L:W
Inclusion bodies	Suspension	Mean ± SD Range	987 ± 231* 667 - 1367*	892 ± 225** 567 - 1300**	
Virus in outer membrane inside the inclusion body	Suspension	Mean ± SD Range	362 ± 22 342 - 383	81 ± 12 67 - 92	4.5 3.7 - 5.1
		Mean of a single virus	-	-	-
		Mean of a bundle	-	-	-
	Thin Section	Range of a single virus	-	58	-
		Range of a bundle	-	-	-
Virus in outer membrane liberated from inclusion body	Suspension	Mean ± SD Range	367 ± 15 358 - 392	90 ± 4 83 - 92	4.0 3.9 - 4.3
		Range of a single virus	-	-	-
		Range of a bundle	-	-	-
Virus in inner membrane outside the inclusion body	Suspension	Mean ± SD Range	427 ± 27 358 - 475	63 ± 3 58 - 67	6.8 6.1 - 8.0
Virus in inner membrane inside the inclusion body	Thin Section	Range	-	25 - 33	-

Table 6.3.1. (Continued)

Material	Treatment (Source)	Type of measurement	Granulosis virus of Heliothis armigera		
			Length nm	Width nm	Ratio L:W
Inclusion bodies	Suspension	Mean ± SD Range	473 ± 51 400 - 542	206 ± 20 175 - 241	2.2 2.0 - 2.7
Virus in outer membrane inside the inclusion body	Suspension	Mean ± SD Range	397 ± 70 300 - 400	72 ± 10 60 - 80	4.6 2.9 - 6.0
		Mean of a single virus	-	-	-
		Mean of a bundle	-	-	-
	Thin Section	Range of a single virus	-	50 - 67	-
		Range of a bundle	-	-	-
Virus in outer membrane liberated from inclusion body	Suspension	Mean ± SD Range	378 ± 11 360 - 390	84 ± 5 75 - 90	4.5 4.0 - 5.2
		Range of a single virus	-	-	-
		Range of a bundle	-	-	-
Virus in inner membrane outside the inclusion body	Suspension	Mean ± SD Range	443 ± 16 420 - 470	64 ± 6 58 - 75	7.0 6.0 - 7.7
Virus in inner membrane inside the inclusion body	Thin Section	Range	-	30 - 40	-

SD = Standard Deviation.

L:W = Length:Width.

* Distance from vertex to opposite vertex in the hexagon.

** Distance from side to opposite side in the hexagon.

6.4. DISCUSSION

A. Shape and size of the inclusion bodies. The observations made with the light microscope were sufficient to give some information on the shape and the size of the polyhedra but the finer details could best be recorded by electron microscopy. The granulosis capsules were easily observed under the ordinary light microscope and did not require dark field or phase contrast microscopy, as stated by Huger (1963). This may be due to the larger dimensions of the capsules which compare well with the longest in the list given by Huger (1963).

The shape and the size of the inclusion bodies was found characteristic enough in the present study to serve as a criterion for identification and classification. Thus, not only could the granulosis capsules be distinguished from the polyhedra, but the two species of polyhedra could also be differentiated from each other. The shape and size as well as the number of viruses occluded in each polyhedrosis and granulosis inclusion body varies with the group. Some polyhedra are triangular, square or irregular (Bergold, 1963a) but the shape of the nuclear-polyhedron of Colias electo and Heliothis zea was found to be roughly hexagonal. It is unlikely that the polyhedra were distorted in preparation for

light or electron microscopy since the shape was the same following the differing preparative techniques. Gregory et al. (1969) who studied the nuclear-polyhedrosis virus of Heliothis zea, also reported irregular 6-sided inclusion bodies using replica and shadowing techniques. No description of the polyhedra of Colias electo virus has been found in the literature, but the related American species, Colias eurytheme, is reported to vary in shape from the triangular to the hexagonal (Steinhaus, 1948). Steinhaus (1948) and Mathad et al. (1968) reported variations in shape and size of polyhedra in different cells of the same host but found it fairly uniform in the nuclei of individual cells. Bergold (1953) on the other hand, reported variations even within one nucleus. Smith (1967) claimed that polyhedra isolated from many hosts of the same species were fairly constant in shape and size; this was also found in the present work. The shape of a polyhedron is known to be determined by the virus and this has been used as a marker in cross infectivity and double infection tests (Hukuhara and Hashimoto, 1966).

Greater variations were found in the size of the polyhedra studied than in their shape. According to the literature, these variations may be due to differences in incubation period (Aruga et al., 1963), in maturity of polyhedra (Steinhaus and Dineen, 1960), in number of occluded virus particles (Hughes, 1950) and in the physiological state of the host (Aizawa and Furuta, 1962). Despite the variations found in the present study, the majority of the polyhedra of each species were of average size but there was a significant difference between the average size of the two

species. The average size of the polyhedra of Heliothis zea, for example, is in accord with other studies on the same virus (Bergold and Ripper, 1957; Gregory et al., 1969). From this consistency it may be assumed that the average size, in addition to the shape, is characteristic of the virus and this was used in Chapter 3 to distinguish between the induced and the inducing polyhedra in the silkworm (3.3.3.).

Despite the irregular shape of the polyhedron, reports in the literature seldom state how the size is measured. In this study, measurements were made from each vertex to the opposite vertex and from each side to the opposite side of the hexagon and the results averaged. This is believed to make comparison in relative size more accurate as undoubtedly some standardization is necessary.

In the case of the granulosis of Heliothis armigera, the shape of the capsules was clearly ovocylindrical, a feature of most granulosis capsules described in the literature with the exception of the cubic capsules isolated by Stairs (1964). A certain variation in shape and size among the granulosis capsules of Heliothis armigera is apparently common in each individual host (Huger, 1963). The occurrence of different outlines in cross sections which was found in this study, was also observed by Bergold (1963b). The presence of virus particles in folded form inside the outer membrane (Fig. 77) may be explained by compression within a short capsule (Fig. 68S), a suggestion supported by the fact that no short virus particles were seen. The presence of long virus capsules, approximately double the length of the average capsule may be the result of two

capsules fused end to end (Steinhaus et al., 1949). Steinhaus and Marsh (1960) and Sidor and Krstić (1969), on the other hand, suggested that they are the result of a virus chain enveloped in one long capsule. The latter explanation seems more likely since in the present study no lines of fusion within the long capsules were seen whereas long virus particles were observed (Fig. 82).

The U-shaped capsules (Fig. 68U) are thought to be a result of breakage across the capsule. Some authors (Smith et al., 1964; Smith and Brown, 1965a; Vago and Bergoin, 1968) have suggested that the capsule is built of a U-shaped structure and a cap which opens to release the virus particle. If this is so, a fairly uniform size of U-shaped capsules would be expected. In fact different sizes were observed in the present study and therefore the breakage is more likely to be a random procedure rather than being dependent upon a specific opening mechanism. Three other ways of releasing the virus particles were observed in this study. The possibility exists that the U-shaped capsules represent defective capsules (Arnott and Smith, 1969) or incomplete ones, since according to Hughes (1952), the capsule formation progresses from one end of the particle to the other.

The spherical capsules (Fig. 68H) probably resulted from cross rupture near one end of the capsule where the smaller portion has rotated through 90°, thus exposing either a hollow core or a spherical end. Since no spherical virus particles were seen, the idea of Bergold (1950) that the spheres represent a developmental stage seems to be unlikely. This is in accordance with Hughes (1952) who found the spheres only after alkali treatment and never during stages

of development. The wheat-like capsules are a result of a longitudinal split which divides the capsule along the long axis (Figs. 72; 73).

B. The inclusion body membrane. Prior to alkali treatment the polyhedra appeared very dark and opaque in the electron microscope even when the particles were unstained; the granulosis capsules were also very opaque. Consequently neither show any lattice structure and virions may rarely be seen inside the inclusion bodies. This opacity may be explained by the presence of a membrane around the inclusion body. In the polyhedra of Colias electo and Heliothis zea this membrane was seen in negatively stained preparations as a thin structure and in thin sections it appeared as a layer showing a strong affinity for stain. Hughes (1950) believed that this membrane was an artifact due to a layer of denatured protein resulting from the alkali treatment. Morgan et al. (1955, 1956), Bergold (1963a) and Smith (1967) also believed that the structure was an artifact since it could not be detected by electron microscopy of thin sections. On the other hand, Ponsen et al. (1964) stated that this membrane, like the inner membrane of the virus, is too thin to be detected in thin sections. Entwistle and Robertson (1968), however, showed a boundary with a great affinity for stain which, when collapsed, was recognised as a membrane in thin sections. Structures observed in preparations of the nuclear-polyhedra of Heliothis zea and Colias electo (Fig. 41) support the assumption that the outer layer is in fact a membrane. The fact that a membrane was noticed around

polyhedra even before alkali treatment suggests that it has not resulted from alkali denaturation.

In suspension, the granulosis capsules showed a double membrane (Fig. 65) thicker than the monolayer membrane of the polyhedra. It could not be seen in thin sections and may have been destroyed by the preparative procedures. The occurrence of a membrane around the granulosis capsules was mentioned by Smith and Brown (1965b) and Smith (1967). Since not all polyhedra and capsules of different species are enclosed in a membrane, it is suggested that its presence or absence may serve as a taxonomic criterion.

C. The occlusion of virus particles in the inclusion body. The difference in number of virus particles in the inclusion body is one of the basic distinguishing features between polyhedra and granulosis capsules. The granulosis virus of Heliothis armigera like other granulosis viruses has one virus particle per capsule. Occasionally two rods are reported in other species (Bergold, 1963b) but the appearance of folded virus particles (Fig. 77) and wheat-like capsules (Fig. 72) may explain how misinterpretations arise regarding the presence of more than one virus particle per capsule. This was also pointed out by Huger (1963).

The polyhedra occlude numerous virus particles occurring either singly, like the nuclear-polyhedra of Heliothis zea, or in bundles where a few virus particles in inner membranes share a common outer membrane such as the polyhedra of Colias electo. In the latter species, single particles did occur, but the greatest number appeared in bundles which contained

2 to 8 virus particles. In thin sections the exact number of virus particles per bundle could readily be seen. In negatively stained polyhedral suspensions the bundles were distinguished from the singly occluded particles by width and only after degradation of the outer membrane was the number of virus particles per bundle revealed.

The consistent occurrence of only singly occluded virus particles in Heliothis zea polyhedra and predominance of bundles in the Colias electo polyhedra suggest that this is a characteristic of the virus species and has been used as a marker in cross infection studies of different polyhedra by Tompkins et al. (1969). In Colias electo polyhedra, where single and multiple virus particles were occluded in the outer membrane, it was found that virus particles in each polyhedron appear either predominantly in bundles or singly, indicating that this is a characteristic not only of the species but of each individual polyhedron. This observation has not been encountered in the available literature. However, the number of virus particles per bundle is not constant to the virus or the host, a point supported by Smith (1967).

D. The inclusion body protein. After partial alkali digestion, the polyhedral and the capsular protein exposed a similar regular cubic lattice arrangement. According to Smith (1967), each molecule has only six selective points of attachment which explains the variation in pattern, i.e. the 'regular arrays of spherical density' and the 'dense bands'. The former appeared in suspensions (Figs. 54; 74) and in thin sections resulting from cross section (Fig. 44P) and the

latter were observed in thin oblique sections through the molecular structure (Fig. 56). These descriptive terms for the patterns have also been used by Morgan et al. (1955). The lattice pattern could be seen to persist as the polyhedral protein disintegrated into smaller pieces during digestion. The scarcity of these small pieces of protein lattice in the preparation of Colias electo indicated a higher degree of solubility in alkali of the protein fragments than that of Heliothis zea polyhedral protein.

The granulosis capsules did not disintegrate but either broke into two parts, or the virus particle escaped through an aperture in the capsule. In both strains of polyhedra and in the granulosis capsules no one particular site seemed more likely to disintegrate than another.

E. The virus particles within the outer membrane. After the virus particles had been released from the inclusion body and had come in contact with the alkali, changes were observed. Some virus particles of the nuclear-polyhedra of Heliothis zea had assumed a horseshoe shape (Figs. 57; 58; 59), observed also by Gregory et al. (1969). Entwistle and Robertson (1968) suggested that the bent particles are less rigid than the straight ones and that the shrinkage of the outer membrane in alkali causes them to assume this shape. The observation of projections on both ends of only the bent particles of Heliothis zea in the present study support this suggestion that the shrinkage of the outer membrane could be the reason for the occurrence of both phenomena, i.e. the bending and the projection or protrusion. The bent particles were erroneously reported by Bergold (1950, 1963b) to be

spherical developmental stages.

Among the virus particles of Colias electo within the outer membrane, no bent particles were seen. The projections, however, were seen on the straight forms either on both ends or on one end and their numbers varied according to the number of occluded virus particles (Figs. 47A, D; 48; 49; 50D). This indicates that the protrusion is related to the occluded virus particle rather than to the outer membrane. Some protrusions were still covered with the flexible outer and inner membranes (Fig. 47A, D) while some had pierced through them (Figs. 49; 50D right low arrow). That the protrusion is a nucleoprotein may be deduced from the fact that loops which are typical of partly uncoiled nucleoprotein (Figs. 64B; 80R) were seen (Figs. 49; 58). Similar loops were reported by Teakle (1969) and Tripconey (1969) who also observed a helical structure in the protrusion. Since the protrusions were never seen in particles still occluded in the inclusion body, a point recorded also by Bergold (1963b), they are believed to have resulted from the shrinkage of the outer membrane in alkali and the consequent expression of the nucleoprotein through the ends, which are mechanically the weakest parts of the membranes. The assumption of Bergold (1963) that these protrusions play a role in the infection mechanism is difficult to accept as these structures were not always present.

The outer membrane of the granulosis virus showed a high resistance to alkali digestion (Chapter 4), a fact recorded also by Bergold (1953) and the inner membrane appeared more rigid than the polyhedral one. These observations may explain why projections or protrusions through the two

membranes were not common among granulosis virus particles. The resistance of the granulosis virus to shrinkage by alkali was further demonstrated by the absence of bent particles. Some were slightly curved (Fig. 74) but similar shapes were found while the particles were still in the capsules (Fig. 68U) and therefore could not be the result of the membrane shrinkage.

The nuclear-polyhedrosis virus of Heliothis zea (Fig. 60A, B) and the granulosis virus of Heliothis armigera (Fig. 79) very often formed spherical sacs when shedding the outer membrane. This is unlike the virus of Colias electo which formed folded arborescent strands (Fig. 47C). The outer membrane of both virus strains was shown to be composed of two layers (Figs. 44L; 60; 75S) as described by Ponsen et al. (1965) and Himeno et al. (1968).

F. The virus particles within the inner membrane. Further digestion in alkali exposed the virus particle within the inner membrane. The released virus appeared longer than the occluded particle in the outer membrane since it was no longer compressed. But even after release, the virus sometimes was curved, with transverse lines which were absent from empty membranes. These lines were also observed by Himeno et al. (1968) and might indicate compression in the Colias electo virus (Figs. 47C, V, M; 50M) or twisting and compression in the Heliothis zea virus (Fig. 61). They were possibly due to the tightly attached inner membrane (Ponsen et al., 1964). That the inner membrane tightly surrounded the virion was demonstrated in the present study by the frequent smaller dimensions of empty membranes compared with

full ones. In thin sections no space was noticed between the inner membrane and its contents, in agreement with Ponsen et al. (1964, 1965). Alternatively, the transverse lines may be a reflection of the coiling of the nucleoprotein (Fig. 64A).

The inner membrane of the nuclear-polyhedrosis and the granulosis viruses was seen to be built of capsomeres as suggested by Kozlov and Alexeenko (1967). This is more readily demonstrated in empty than on full membranes (Figs. 47E; 51; 62; 63; 81). The suggestion of Bergold and Wellington (1954) that the membrane disintegrates to threads, possibly indicating the spiral structure of it, could not be supported in the present study since no threads were observed to originate from membranes. Neither was the hypothesis of Harrap and Juniper (1966) of an assembly of rings to form the membrane supported since no spherical rings were observed.

The two distinct ends of the full or empty membranes were frequently seen and appear to have a strong capacity of attachment as seen in Fig. 51. Although the function of these two ends is yet unknown, they may well play a role in the infection mechanism by attaching themselves to the susceptible cells.

The granulosis virus within the inner membrane differed from the nuclear-polyhedrosis viruses in being more opaque (Figs. 79; 80M). Since the empty inner membranes did not differ in thickness, the particles probably differed in the amount of contained nucleoprotein. Therefore the structures on both ends of the inner membrane were not very different in density from the rest of the virus and were consequently less

easily detectable than in the nuclear-polyhedrosis viruses. When observed on empty membranes, the appearance of the ends of the two types of viruses was similar. The membrane, being more rigid than that of the nuclear-polyhedrosis viruses, sometimes withstood the alkali treatment while its contents were dissolved as in Fig. 78. Dissolution of the central part, leaving the ends and the membranes intact is also shown by Smith and Xeros (1954b). Being more rigid, the inner membrane of the granulosis virus was also less flexible and often broke in the centre releasing the nucleoprotein (Fig. 80R).

G. The nucleoprotein and theories of formation.

The helical structure of the nucleoprotein of the nuclear-polyhedrosis viruses was demonstrated (Figs. 52; 64A). The presence of two uncoiling bands of nucleoprotein, while escaping out of the inner membrane, was also observed (Figs. 52 arrow; 64B). These two findings support the formation model proposed by Kozlov and Alexeenko (1967) showing two bands of nucleoprotein coiled together. The theory of Bergold (1963b) of an assembly of 6-8 subunits across the nucleoprotein cannot be accepted since the latter was never observed to degrade into subunits and was only observed to uncoil. Due to the small size, it could not be determined whether each nucleoprotein band was a double helix as proposed by Onodera et al. (1965). Neither could the assembly of protein subunits around the nucleic acid in a fashion similar to tobacco mosaic virus (Franklin and Klug, 1956; Kreig, 1961, in Smith, 1967) be confirmed.

The nucleoprotein in the granulosis virus was observed to uncoil (Fig. 80R) but a helix was not observed. Very little is known about the internal structure of granulosis viruses and only vague demonstrations of helical structure have appeared in the literature (Smith and Hills, 1962, in Smith, 1967; Sidor and Krstić, 1969).

The granulosis virus is believed to follow the same model of formation as the nuclear-polyhedrosis virus (Smith, 1967). An alternative way of replication through breakage of DNA containing filaments is known to occur in granulosis viruses (Smith and Brown, 1965b; Arnott and Smith, 1969). The long virus particle (Fig. 82) may represent the linear form of these filaments (Steinhaus and Marsh, 1960). Often, however, they are known to be either branched (Smith and Brown, 1965a) or linear with breaks (Smith et al., 1964). The unusual club-shaped cavities in the capsules forming 'head' and 'tail' (Figs. 70; 71) possibly occluded a filament in the tail emerging from a normal virus particle in the head. Similar structures were shown by Smith and Rivers (1956) and Smith and Brown (1965a, b).

In this study, the sequence of occlusion of the nucleoprotein by the inner membrane followed by the outer membrane and later by the inclusion body was conversely demonstrated by stages of alkali degradation of the studied viruses.

Non-viral structures similar to the virus-like structure in Fig. 83 have been reported by Ignoffo (1968), Sidor and Krstić (1969, Fig. 6) and Smith and Brown (1965b) and erroneously thought to represent the nucleoprotein of the virus. Summers and Paschke (1968) observed similar

structures in a granulosis preparation but challenged its viral origin. Gregory et al. (1969) suggested that the virus-like structures might be bacterial rapidosomes. Both types of thin and thick structures were reported by Pate et al. (1967) to be rapidosomes of different bacteria, resulting from the degradation of the cell wall. The fact that in the present study these non-viral structures were found after treatments of alkali, ultrasound and caesium chloride, points to their nature as break-down products. They were also found in preparations of cytoplasmic-polyhedrosis virus which is spherical, indicating that they are not connected with the long bands of virus nucleoprotein.

H. The size of the virus particles. The shape of the virus particles within the outer and inner membrane of the three viruses studied was found to be basically the same. Their size range, however, was typical to each virus species (Table 6.3.1.).

In suspensions, when comparing the measurements of the virus particles within the outer membrane inside and outside the inclusion body, there was not a significant difference, indicating no or little influence of the alkali and the negative stain on the size of the liberated virus particles. Shrinking and destruction by alkali and the possible influence of the negative stain, as reported by Bergold (1963b) was not encountered. Swelling during exposure to alkali and flattening during drying on the grid (Ponsen et al., 1964; Entwistle and Robertson, 1968) was not observed. In thin sections the lesser width of the virus particles compared with that of particles in suspension can therefore be

attributed to shrinkage during fixation, dehydration and embedding procedures (Morgan et al., 1955).

The dimensions of single virus particles were fairly uniform. The variations in length and, to a lesser extent, in width were due to different degrees of shrinkage of the outer and the inner membranes, or to different degrees of twisting and compression while in the inner membrane. The length:width ratio changed accordingly. The mean values and the range of dimensions, however, were typical for each virus and can serve as a criterion for identification and classification. For purposes of comparison a standardization of measurements is necessary, since in the literature very few authors state whether the virus particle was measured within its outer membrane or its inner membrane.

CHAPTER 7

CONCLUSIONS

Three insect viruses were compared and contrasted. The morphology and the fine structure of the inclusion bodies and virus particles isolated from Colias electo and Heliothis zea, although strikingly similar, could be distinguished from one another. On the other hand, while occluded within its capsule, the granulosis virus of Heliothis armigera was morphologically quite distinct. The released virus particles of Heliothis armigera, however, resembled very closely those of the other two viruses.

All three inclusion bodies were shown to have a similar pattern of assembly of the polyhedral or capsular protein molecules in the form of a regular cubic paracrystalline lattice. The solubility in alkali of this proteinaceous lattice varied considerably with the virus strain.

The two polyhedra were roughly hexagonal and occluded numerous virus particles, but their respective sizes differed to the extent that the polyhedral hexagons of Colias electo gave average dimensional measurements of 1835 $\pm$ 616 or 1632 $\pm$ 590 nm, while those of Heliothis zea measured 987 $\pm$ 231 or 892 $\pm$ 225 nm. The granulosis capsules of Heliothis armigera were of quite different shape being ovocylindrical, containing only one occluded virus particle and measuring 473 $\pm$ 51 in length and 206 $\pm$ 20 nm in width.

The liberated virus particles of the three species were rod-shaped and consisted of helical nucleoprotein surrounded by an inner and an outer membrane. The inner membrane was seen to have a fine structure of regularly placed capsomeres with dissimilar structures at either end. These terminal structures, the 'claw-setting' or the 'nipple' were thought to have some role in attachment and the initiation of infection.

There were, however, some characteristic differences among the virus particles. Within the outer membrane, the virus of Colias electo appeared predominantly as bundles of 2-8 rod-shaped particles with only an occasional single particle. The other two viruses appeared only as single particles within the outer membrane.

Projections and protrusions from the outer membrane were variable but not specific or constant for each virus strain. The frequency of these projections and protrusions in the virus particles of Colias electo and their appearance only in the bent particles of Heliothis zea, compared with their relative rarity in the granulosis particles, suggests that the nature of the membrane accounts for their presence. Differences in stability of the membranes, as demonstrated by alkali digestion, are believed to bring about varying degrees of shrinkage of the outer membrane, thus causing the nucleoprotein to be squeezed out in the form of projections or protrusions.

The mean measurements of the length and width of the virus particles within the membranes were calculated. The particles were invariably found to be greater in width and shorter in length when they were within the outer membrane

than when they were within the inner membrane alone. This clearly showed that the outer membrane caused some compression of the virus particle, an observation which was repeatedly confirmed by electron microscopy.

Table 7.1. Mean measurements - length and width - of the virus particles.

	Nuclear-polyhedrosis virus of *Colias electo*	Nuclear-polyhedrosis virus of *Heliothis zea*	Granulosis virus of *Heliothis armigera*
In outer membrane	313 ± 19 x 104 ± 19 nm	367 ± 15 x 90 ± 4 nm	378 ± 11 x 84 ± 5 nm
In inner membrane	350 ± 30 x 47 ± 4 nm	427 ± 27 x 63 ± 3 nm	443 ± 16 x 64 ± 6 nm

In attempts to purify the inclusion bodies and the virus particles, difficulties were encountered which may be related to the nature of the initial material. In two instances this material consisted of putrefying insect tissues heavily contaminated with bacteria; in the third instance, this was the commercial preparation which consisted of a powder matrix in which the inclusion bodies were closely held. A wide variety of standard techniques were tried with varying degrees of success. These were differential centrifugation, shaking with the organic solvent fluorocarbon, treatment with ultrasound and zone electrophoresis in a sucrose density gradient. The techniques were used in various combinations. These treatments gave satisfactory partially purified end-products, but the loss of material during purification was often considerable. Techniques which were used with success

in the purification of inclusion bodies were not always applicable to purification of the virus particles because of their lesser stability.

It was important to have a safe method of storing inclusion bodies as the source of infected larvae was seasonal. Fortunately, the partially purified polyhedra of Colias electo and the granulosis capsules of Heliothis armigera withstood lyophilization and could be safely stored in the dried state at 4°C. The viral material stored in this manner proved useful as a standard stock material.

No histopathological investigations were undertaken to establish in which tissues, cells and intracellular situations the inclusion bodies occurred. Neither were the early stages of infection studied. The release of virus particles from the inclusion bodies in vitro, which may well be similar in vivo, was, however, followed in some detail. This release was achieved by controlling the digestion of the inclusion bodies with weak alkali through varying the molarity of the sodium carbonate and the duration of exposure according to the stability of the inclusion bodies. Disintegration of the inclusion body protein resulted in the release of virus particles from the proteinaceous matrix in which they were embedded. Partial purification of the virus particles was achieved by differential centrifugation followed by electrophoresis in a sucrose density gradient. The latter technique also enabled separation of the virus particles within the outer membrane from those within the inner membrane alone. The release of the virus particle from the outer membrane was shown to be a gradual process

due to the graded loss of surface properties which determined the mobility in the electric field. This process was also observed by electron microscopy but the morphology of the outer membrane and the manner of its digestion was different for the virus of Colias electo as compared to the other two species. The morphology of the inner membrane and its manner of disintegration, however, was very similar for each of the three species, although the membrane of the granulosis virus appeared more rigid.

The electrophoresis mobility of the inclusion bodies and the virus particles was found to be of importance in two ways. In the first, the mobility of the viral elements relative to extraneous non-viral substances aided the purification of the former to a degree not possible with standard methods of fractionation. In the second, the mobility of the viral elements relative to that of a reference substance, phenol red, provided a simple biophysical measurement which was found useful in identification of a species and its distinction from others. This ratio, expressed as R_{ϕ}, was useful in recording differences between the inclusion bodies, where these differences were significant. The R_{ϕ} values for the polyhedra of Colias electo and of Heliothis zea were 0.91 and 0.73, respectively, while that for the granulosis capsules of Heliothis armigera was 0.88. The smaller differences between the R_{ϕ} values for the virus particles were neither distinctive nor reproducible on account of the varying amount of surface membrane which had been lost or remained attached to the particles.

Certain observations made in the present study may well prove to be important in considering the use of the two South African virus strains for biological control of the lucerne caterpillar, Colias electo, and the bollworm, Heliothis armigera.

The viruses of these two species are both highly pathogenic for their hosts, particularly in the larval stages. The relative pathogenicity, or virulence, of the viruses may be enhanced by conditions of stress as was shown by elevation of the temperature and, to a lesser extent, by spraying the leaves with endospores of Bacillus thuringiensis. Due to their virulence, only a small infective dose of virus may be sufficient to initiate infection.

Thereafter, the infection may be transmitted and maintained through the liberation of infected body contents from dead larvae or by cannibalism of virus-infected material by healthy larvae, as in the case with the bollworm. Transmission from one generation to the next of cytoplasmic-polyhedrosis virus via the eggs was also demonstrated in the silkworm. This is a well known phenomenon among granulosis and nuclear-polyhedrosis viruses as has already been shown by Smith and Rivers (1956) and Bird (1961), respectively.

Stability of the inclusion bodies, particularly the capsules of the granulosis virus, may be expected to have considerable importance in biological control. In this study, the persistence of the granulosis capsules of Heliothis armigera has been demonstrated in the avian faeces of the cattle egret, thus favouring transmission to other geographical areas. The higher stability also accounts for

the persistence of other inclusion bodies in the soil and on foliage of plants from one season to the next as has been recorded by David and Gardiner (1966) and Jaques (1966), respectively.

Another conclusion of importance arising from this study is the need for a more specific system of classification of insect viruses. That in use at present is too broad and at most tentative because the taxonomic groupings depend more on the effects produced by the viruses in a particular host than on the fundamental properties of the viruses themselves.

Viruses of the genus Borrelinavirus are the nuclear-polyhedrosis viruses which propagate in mesodermal and ectodermal tissues. By contrast, the Birdiavirus genus includes those nuclear-polyhedrosis viruses which replicate only in endodermal structures (Ignoffo, 1968). On the basis of morphological and symptomatological data derived from this study, it appears that the viruses isolated from the South African lucerne caterpillar, Colias electo, and from the American bollworm, Heliothis zea, fall into the group designated Borrelinavirus.

Viruses of the genus Bergoldiavirus are rod-shaped and occluded singly in an ovocylindrical capsule and propagated in the cell nucleus and cytoplasm, in contrast with those that replicate only in the nucleus of the infected cell and grouped together as the Steinhausiavirus genus (Ignoffo, 1968). As histological examinations of the infected cells were not made in this study, it has not been possible to decide in which of these two broad groups the granulosis virus of

Heliothis armigera should be placed.

It seems likely, however, that future systems of nomenclature and taxonomy will make greater use of the fundamental properties of the inclusion bodies and the virus particles in the form of morphological, biophysical and biological measurements in addition to information on the complex relationships between the host and the infecting virus.

It is hoped that the information presented in this thesis on three insect viruses, two of which are of local origin, will contribute something worthwhile to the understanding of the significance of these agents in agriculture.

Since it is believed that the arbitrary application of virus suspensions to crops is unlikely to achieve success in the biological control of agricultural pests, it is felt that the identification, differentiation and selection of suitable strains of virus with particular reference to stability, pathogenicity, transmissibility and high lethal efficiency will lead the way to future programmes of effective and specific biological control.

REFERENCES

Adams, J.R., and Wilcox, T.A. 1968. Histopathology of the almond moth, Cadra cautella, infected with a nuclear-polyhedrosis virus. J. Invertebrate Pathol., 12, 269-274.

Aizawa, K. 1962. Antiviral substance in the gut juice of the silkworm, Bombyx mori (Linnaeus). J. Insect Pathol., 4, 72-76.

Aizawa, K. 1963. The nature of infections caused by nuclear-polyhedrosis viruses. In "Insect Pathology - An Advanced Treatise" (E.A. Steinhaus, ed.), Vol. 1, pp. 381-412. Academic Press, New York.

Aizawa, K., and Furuta, Y. 1962. Multiplication of nuclear and cytoplasmic polyhedrosis viruses in starved larvae of the silkworm, Bombyx mori (Linnaeus). J. Insect Pathol., 4, 465-468.

Arnott, H.J., and Smith, K.M. 1969. Ultrastructural observations on the branched rods associated with some insect granuloses. J. Invertebrate Pathol., 13, 345-350.

Aruga, H. 1963. Induction of virus infections. In "Insect Pathology - An Advanced Treatise" (E.A. Steinhaus, ed.), Vol. 1, pp. 499-530. Academic Press, New York.

Aruga, H., Hukuhara, T., Fukuda, S., and Hashimoto, Y. 1963. Interference between cytoplasmic polyhedrosis viruses of the silkworm, Bombyx mori (Linn.), and of the pine caterpillar, Dendrolimus spectabilis (Butler). J. Insect Pathol., 5, 415-421.

Bedford, E.C.G. 1961. Recent biological control projects and the further application of biological control. Symp. Entomol. Problems. Tech. Commun., 12, 188-195. Dept. Agr. Tech. Services, Pretoria.

Benz, G. 1963. The nature of nuclear-polyhedrosises. In "Insect Pathology - An Advanced Treatise" (E.A. Steinhaus, ed.), Vol. 1, pp. 299-338. Academic Press, New York.

Bergold, G.H. 1950. The multiplication of insect viruses as organisms. Canad. J. Res., Ser. E, 28, 5-11.

Bergold, G.H. 1953. Insect viruses. Advances Virus Res. 1, 91-139.

Bergold, G.H. 1959. Purification of insect virus inclusion bodies with fluorocarbon. J. Insect Pathol., 1, 96-100.

Bergold, G.H. 1963a. The nature of nuclear-polyhedrosis viruses. In "Insect Pathology - An Advanced Treatise" (E.A. Steinhaus, ed.), Vol. 1, pp. 413-456. Academic Press, New York.

Bergold, G.H. 1963b. Fine structure of some insect viruses. J. Insect Pathol., 5, 111-128.

Bergold, G.H. 1964. Insect viruses. In "Techniques In Experimental Virology" (R.J.C. Harris, ed.), pp. 111-144. Academic Press, New York.

Bergold, G.H., and Ripper, W.E. 1957. The polyhedral virus of Heliothis armigera (Hbn.)(Lepidoptera: Noctuidae). Nature, 180, 764-765.

Bergold, G.H., and Suter, J. 1959. On the structure of cytoplasmic polyhedra of some Lepidoptera. J. Insect Pathol., 1, 1-14.

Bergold, G.H., and Wellington, E.F. 1954. Isolation and chemical composition of membranes of an insect virus and their relation to the virus and polyhedral bodies. J. Bacteriol., 67, 210-216.

Bird, F.T. 1952. On the multiplication of an insect virus. Biochim. Biophys. Acta, 8, 360-368.

Bird, F.T. 1959. Polyhedrosis and granulosis viruses causing single and double infections in the spruce budworm, Choristoneura fumiferana Clemens. J. Insect Pathol., 1, 406-430.

Bird, F.T. 1961. Transmission of some insect viruses with particular reference to ovarial transmission and its importance in the development of epizootics. J. Insect Pathol. 3, 352-380.

Bird, F.T. 1963. On the development of granulosis viruses. J. Insect Pathol., 5, 368-376.

Blitz, J. 1967. The properties of ultrasonic waves. In "Ultrasonic Techniques In Biology And Medicine" (B. Brown and D. Gordon, ed.), pp. 1-26. London Iliffe Books, London.

Bradley, D.E. 1965. The preparation of specimen support films. In "Techniques For Electron Microscopy", 2nd ed., (D.H. Kay, ed.), pp. 58-74. Blackwell Scientific Publications, Oxford.

Brakke, M.K. 1953. The combination of zone electrophoresis and density gradient centrifugation in purification of some unstable plant viruses. Phytopathology, 43, 467.

Brakke, M.K. 1955. Zone electrophoresis of dyes, proteins and viruses in density-gradient columns of sucrose solutions. Arch. Biochem. Biophys., 55, 175-190.

Brenner, S., and Horne, R.W. 1959. A negative staining method for high resolution electron microscopy of viruses. Biochim. Biophys. Acta, 34, 103-110.

Brenner, S., Streisinger, G., Horne, R.W., Champe, S.P., Barnett, L., Benzer, S., and Rees, M.W. 1959. Structural components of bacteriophage. J. Molec. Biol., 1, 281-292.

Bucher, G.E. 1963. Ultrasonic cleaning of spores of an obligate bacterial pathogen. J. Insect Pathol., 5, 427-433.

Bucher, G.E. and Harris, P. 1968. Virus diseases and their interaction with food stress in Calophasia lunula. J. Invertebrate Pathol., 10, 235-244.

Canerday, T.D., and Arant, F.S. 1968. Pathogen-host-environment relationships in a cabbage looper nuclear polyhedrosis. J. Invertebrate Pathol., 12, 344-348.

Chapman, J.W., and Glaser, R.W. 1915. A preliminary list of insects which have wilt, with a comparative study of the polyhedra. J. Econ. Entomol., 8, 140-150.

Clark, G.C., and Dickson, C.G.C. 1952. "Some South African Butterflies". Longmans Green and Co., Cape Town.

Common, I.F.B. 1953. The Australian species of Heliothis (Lepidoptera: Noctuidae) and their pest status. Austr. J. Zool., 1, 319-344.

Cramer, R. 1959. Zone electrophoresis of rous sarcoma virus. Nature, 183, 195-196.

Cramer, R., and Stewart, S.E. 1960. Zone electrophoresis studies on hemagglutinin of hemagglutinating and of masked strain of polyoma virus. Proc. Soc. Exp. Biol. Med., 103, 697-700.

Cunningham, J.C. 1968. Serological and morphological identification of some nuclear-polyhedrosis and granulosis viruses. J. Invertebrate Pathol., 11, 132-141.

David, W.A.L., and Gardiner, B.O.C. 1965. The incidence of granulosis deaths in susceptible and resistant Pieris brassicae (Linnaeus) larvae following changes of population density, food, and temperature. J. Invertebrate Pathol., 7, 347-355.

David, W.A.L., and Gardiner, B.O.C. 1966. Persistence of a granulosis virus of Pieris brassicae on cabbage leaves. J. Invertebrate Pathol., 8, 180-183.

David, W.A.L., Gardiner, B.O.C., and Clothier, S.E. 1968. Laboratory breeding of Pieris brassicae transmitting a granulosis virus. J. Invertebrate Pathol., 12, 238-244.

Doane, C.C. 1967. Bioassay of nuclear-polyhedrosis virus against larval instars of the gypsy moth. J. Invertebrate Pathol., 9, 376-386.

Doane, C.C. 1969. Trans-ovum transmission of a nuclear-polyhedrosis virus in the gypsy moth and the inducement of virus susceptibility. J. Invertebrate Pathol., 14, 199-210.

Doane, C.C. 1970. Primary pathogens and their role in the development of an epizootic in the gypsy moth. J. Invertebrate Pathol., 15, 21-33.

Drummond, D.G. 1950. The practice of electron microscopy. Basic techniques. J. Roy. Microsc. Soc., 70, 17-30.

Entwistle, P.F., and Robertson, J.S. 1968. An unusual nuclear-polyhedrosis virus from larvae of a Hepialid moth. J. Invertebrate Pathol., 11, 487-495.

Estes, Z.E., and Faust, R.M. 1966. Silicon content of intact nuclear polyhedra from the corn earworm, Heliothis zea. J. Invertebrate Pathol., 8, 145-159.

Falcon, L.A., Kane, W.R., Etzel, L.K., and Leutenegger, R. 1967. Isolation of a granulosis virus from the Noctuid Heliothis zea. J. Invertebrate Pathol., 9, 134-136.

Farrant, J.L. 1954. An electron microscopic study of ferritin. Biochim. Biophys. Acta, 13, 569-576.

Faulkner, P., Martin, E.M., Sved, S., Valentine, R.C., and Work, T.S. 1961. Studies on protein and nucleic acid metabolism in virus-infected mammalian cells. 2. The isolation, crystallization and chemical characterization of mouse encephalomyocarditis virus. Biochem. J., 80, 597-605.

Faust, R.M., and Adams, J.R. 1966. The silicon content of nuclear and cytoplasmic viral inclusion bodies causing polyhedrosis in Lepidoptera. J. Invertebrate Pathol., 8, 526-530.

Faust, R.M., and Adams, J.R. 1967. The silicon content of an intact granulosis virus from the salt-marsh caterpillar, Estigmene acrea. J. Invertebrate Pathol., 9, 142-143.

Faust, R.M., and Estes, Z.E. 1965. Nucleic acid composition of the nuclear-polyhedrosis virus from Trichoplusia ni. J. Invertebrate Pathol., 7, 521-522.

Faust, R.M., and Estes, Z.E. 1966. Silicon content of the parasporal crystals bacteria. J. Invertebrate Pathol., 8, 141-144.

Franklin, R.E., and Klug, A. 1956. The nature of the helical groove on the tobacco mosaic virus particle. X-ray diffraction studies. Biochim. Biophys. Acta, 19, 403-416.

Gessler, A.E., Bender, C.E., and Parkinson, M.C. 1956a. A new and rapid method for isolating viruses by selective fluorocarbon deproteinization. Trans. N.Y. Acad. Sci., Ser. 2, 18, 701-703.

Gessler, A.E., Bender, C.E., and Parkinson, M.C. 1956b. Animal viruses isolated by fluorocarbon emulsification. Trans. N.Y. Acad. Sci., Ser. 2, 18, 707-717.

Gitay, H., and Polson, A. 1971. Isolation of granulosis virus from Heliothis armigera and its persistence in avian faeces. J. Invertebrate Pathol., 17, 288-290.

Glauert, A.M. 1965. The fixation and embedding of biological specimens. In "Techniques For Electron Microscopy", 2nd ed., (D.H. Kay, ed.), pp. 166-212. Blackwell Scientific Publications, Oxford.

Glauert, A.M., Rogers, G.E., and Glauert, R.H. 1956. A new embedding medium for electron microscopy. Nature, 178, 803.

Gregory, B.G., Ignoffo, C.M., and Shapiro, M. 1969. Nucleopolyhedrosis of Heliothis: morphological description of inclusion bodies and virions. J. Invertebrate Pathol., 14, 186-193.

Hall, C.E. 1955. Electron densitometry of stained virus particles. J. Biophys. Biochem. Cytol., 1, 1-12.

Hamm, J.J., and Paschke, J.D. 1963. On the pathology of a granulosis of the cabbage looper, Trichoplusia ni (Hübner). J. Insect Pathol., 5, 187-197.

Harrap, K.A., and Juniper, B.E. 1966. The internal structure of an insect virus. Virology, 29, 175-178.

Himeno, M., Yasuda, S., Kohsaka, T., and Onodera, K. 1968. The fine structure of a nuclear-polyhedrosis virus of the silkworm. J. Invertebrate Pathol., 11, 516-519.

Horne, R.W. 1965. The examination of small particles. In "Techniques For Electron Microscopy", 2nd ed., (D.H. Kay, ed.), pp. 311-327. Blackwell Scientific Publications, Oxford.

Horne, R.W., and Wildy, P. 1963. Virus structure revealed by negative staining. Advances Virus Res., 10, 101-170.

Hostetter, D.L., and Biever, K.D. 1970. The recovery of virulent nuclear-polyhedrosis virus of the cabbage looper, Trichoplusia ni, from the feces of birds. J. Invertebrate Pathol., 15, 173-176.

Huger, A. 1963. Granulosis of insects. In "Insect Pathology - An Advanced Treatise" (E.A. Steinhaus, ed.), Vol. 1, pp. 531-575. Academic Press, New York.

Huger, A., and Krieg, A. 1961. Electron microscope investigations on the virogenesis of the granulosis of Choristoneura murinana (Hbn.). J. Insect Pathol., 3, 183-196.

Hughes, K.M. 1950. A demonstration of the nature of polyhedra using alkaline solutions. J. Bacteriol., 59, 189-195.

Hughes, K.M. 1952. Development of the inclusion bodies of a granulosis virus. J. Bacteriol., 64, 375-380.

Hughes, D.E., and Nyborg, W.L. 1962. Cell disruption by ultrasound. Science, 138, 108-114.

Hukuhara, T. 1962. Generation to generation transmission of the cytoplasmic polyhedrosis virus of the silkworm, Bombyx mori L. J. Insect Pathol., 4, 132-135.

Hukuhara, T., and Hashimoto, Y. 1966. Serological studies of the cytoplasmic- and nuclear-polyhedrosis viruses of the silkworm, Bombyx mori. J. Invertebrate Pathol., 8, 234-239.

Huxley, H.E. and Zubay, G. 1960. The structure of the protein shell of turnip yellow mosaic virus. J. Molec. Biol., 2, 189-196.

Ignoffo, C.M. 1965a. The nuclear-polyhedrosis virus of Heliothis zea (Boddie) and Heliothis virescens (Fabricius). I. Virus propagation and its virulence. J. Invertebrate Pathol., 7, 209-216.

Ignoffo, C.M. 1965b. The nuclear-polyhedrosis virus of Heliothis zea (Boddie) and Heliothis virescens (Fabricius). II. Biology and propagation of diet-reared Heliothis. J. Invertebrate Pathol., 7, 217-226.

Ignoffo, C.M. 1966a. Effects of age on mortality of Heliothis zea and Heliothis virescens larvae exposed to a nuclear-polyhedrosis virus. J. Invertebrate Pathol., 8, 279-282.

Ignoffo, C.M. 1966b. Effects of temperature on mortality of Heliothis zea larvae exposed to sublethal doses of a nuclear polyhedrosis virus. J. Invertebrate Pathol., 8, 290-292.

Ignoffo, C.M. 1968. Viruses - living insecticides. Curr. Top. Microbiol. Immun., 42, 129-167.

Ignoffo, C.M., Chapman, A.J., and Martin, D.F. 1965. The nuclear-polyhedrosis virus of Heliothis zea (Boddie) and Heliothis virescens (Fabricius). III. Effectiveness of the virus against field populations of Heliothis on cotton, corn and sorghum. J. Invertebrate Pathol., 7, 227-235.

Jaques, R.P. 1961. The influence of physical stress on growth and nuclear polyhedrosis of Trichoplusia ni (Hübner). J. Insect Pathol., 3, 47-54.

Jaques, R.P. 1962. Stress and nuclear polyhedrosis in crowded populations of Trichoplusia ni (Hübner). J. Insect Pathol., 4, 1-22.

Jaques, R.P. 1969. Leaching of the nuclear-polyhedrosis virus of Trichoplusia ni from soil. J. Invertebrate Pathol., 13, 256-263.

Jaques, R.P., and Huston, F. 1969. Tests on microbial decomposition of polyhedra of the nuclear-polyhedrosis virus of the cabbage looper, Trichoplusia ni. J. Invertebrate Pathol., 14, 289-290.

Kellenberger, E., Ryter, A., and Sechaud, J. 1958. Electron microscope study of DNA-containing plasms. II. Vegetative and mature phage DNA as compared with normal bacterial nucleotides in different physiological states. J. Biophys. Biochem. Cytol., 4, 671-678.

Kozlov, E.A., and Alexeenko, I.P. 1967. Electron-microscope investigation of the structure of the nuclear-polyhedrosis virus of the silkworm, Bombyx mori. J. Invertebrate Pathol., 9, 413-419.

Lounsbury, C.P. 1913. Caterpillar wilt disease. Agr. J. Union S. Africa, 5, 448-452.

Luft, J.H. 1961. Improvements in epoxy resin embedding method. J. Biophys. Biochem. Cytol., 9, 409-414.

Mally, C.W. 1908. Cutworms. Poisoned bait remedy. Agr. J. Cape of Good Hope, 33, 628-635.

Martignoni, M.E. 1964. Progressive nucleopolyhedrosis in adults of Peridroma saucia (Hübner). J. Insect Pathol., 6, 368-372.

Mathad, S.B., Splittstoesser, C.M., and McEwen, F.L. 1968. Histopathology of the cabbage looper, Trichoplusia ni, infected with a nuclear polyhedrosis. J. Invertebrate Pathol., 11, 456-464.

Michelbacher, A.E., and Smith, R.F. 1943. Some natural factors limiting the abundance of the alfalfa butterfly. Hilgardia, 15, 369-397.

Miyajima, S., Kimura, I., and Kawase, S. 1969. Purification of a cytoplasmic-polyhedrosis virus of the silkworm, Bombyx mori. J. Invertebrate Pathol., 13, 296-302.

Morgan, C., Bergold, G.H., Moore, D.H., and Rose, H.M. 1955. The macromolecular paracrystalline lattice of insect viral polyhedral bodies demonstrated in ultrathin sections examined in the electron microscope. J. Biophys. Biochem. Cytol., 1, 187-190.

Morgan, C., Bergold, G.H., and Rose, H.M. 1956. Use of serial sections to delineate the structure of Prothetria dispar virus in the electron microscope. J. Biophys. Biochem. Cytol., 2, 23-28.

Morris, O.N. 1962. Quantitative infectivity studies on the nuclear polyhedrosis of the western oak looper, Lambdina fiscellaria (Hulst). J. Insect Pathol., 4, 207-215.

Nayar, J.K., and Fraenkel, G. 1963. Practical methods of year-round laboratory rearing of the silkworm Bombyx mori (L.) (Lepidoptera, Bombycidae). Ann. Entomol. Soc. Amer., 56 (1), 122-123.

Neilsen, M.M. 1965. Effects of a cytoplasmic polyhedrosis virus on adults Lepidoptera. J. Invertebrate Pathol., 7, 306-314.

Nel, J.J.C. 1958. Bionomic and taxonomic studies of Heliothis armigera (Hub) (Lepidoptera: Agrotidae). M.Sc. thesis, University of Stellenbosch.

Onodera, K., Komano, T., Himeno, M., and Sakai, F. 1965. The nucleic acid of nuclear-polyhedrosis virus of the silkworm. J. Molec. Biol., 13, 532-539.

Palade, G.E. 1952. A study of fixation for electron microscopy. J. Exp. Med., 95, 285-297.

Pate, J.L., Johnson, J.L., and Ordal, F.J. 1967. The fine structure of Chondrococcus columnaris. II. Structure and formation of rhapidosomes. J. Cell. Biol., 35, 15-35.

Peacocke, A.R., and Pritchard, N.J. 1968. Some biophysical aspects of ultrasound. Progr. Biophys., 18, 187-208.

Pease, D.C. 1964. "Histological Techniques For Electron Microscopy", 2nd ed. Academic Press, New York.

Philipson, L. 1967. Water-organic solvent phase systems. In "Methods In Virology" (K. Maramorosch and H. Koprowski, ed.), Vol. 2, pp. 235-244. Academic Press, New York.

Pimentel, D., and Shapiro, M. 1962. The influence of environment on a virus-host relationship. J. Insect Pathol., 4, 77-87.

Polson, A., and Cramer, R. 1958. Zone electrophoresis of type 1 poliomyelitis virus. Biochim. Biophys. Acta, 29, 187-192.

Polson, A., and Deeks, D. 1962. Zone electrophoresis of enteroviruses. J. Hyg., 60, 217-234.

Polson, A., and Levitt, J. 1963. Density determination in a preformed gradient of caesium chloride. Biochim. Biophys. Acta, 75, 88-95.

Polson, A., and Russell, B. 1967. Electrophoresis of viruses. In "Methods in Virology" (K. Maramorosch and H. Koprowski, ed.), Vol. 2, pp. 391-426. Academic Press, New York.

Ponsen, M.B., Henstra, S., and Van der Scheer, C. 1964. Electron microscope observations of nuclear polyhedra from Malacosoma neustria (Lepidoptera: Lasiocompidae). Neth. J. Plant Pathol., 70, 101-104.

Ponsen, M.B., Henstra, S., and Van der Scheer, C. 1965. Electron microscope observations on the structure of membranes from nuclear polyhedral viruses. Neth. J. Plant Pathol., 71, 20-24.

Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell. Biol., 17, 208-212.

Sabatini, D.D., Bensch, K., and Barrnett, R.J. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J. Cell. Biol., 17, 19-58.

Shapiro, M., and Ignoffo, C.M. 1969. Nuclear polyhedrosis of Heliothis : stability and relative infectivity of virions. J. Invertebrate Pathol., 14, 130-134.

Sidor, Č., and Krstić, R. 1969. Electron-microscope studies of a granulosis virus of Pygaera anastomsis. J. Invertebrate Pathol., 13, 19-24.

Smirnoff, W.A. 1967. Influence of light on the development of a nuclear-polyhedrosis of Neodiprion swainei. J. Invertebrate Pathol., 9, 269-271.

Smit, B. 1936. The insect pests of lucerne in South Africa. Union S. Africa Dept. Agr. Forest. Bull., 170, 84-88.

Smith, K.M. 1963. The cytoplasmic virus diseases. In "Insect Pathology - An Advanced Treatise" (E.A. Steinhaus, ed.), Vol. 1, pp. 457-497. Academic Press, New York.

Smith, K.M. 1967. "Insect Virology". Academic Press, New York.

Smith, K.M., and Brown, R.M. 1965a. Replication cycle of an insect granulosis virus. Nature, 207, 106-107.

Smith, K.M., and Brown, R.M. 1965b. A study of the long virus rods associated with insect granuloses. Virology, 27, 512-519.

Smith, K.M., and Rivers, C.F. 1956. Some viruses affecting insects of economic importance. Parasitology, 46, 235-242.

Smith, K.M., and Wyckoff, R.W.G. 1950. Structure within polyhedra associated with insect virus diseases. Nature, 166, 861-862.

Smith, K.M., and Xeros, N. 1954a. An unusual virus disease of a Dipterous larva. Nature, 173, 866-867.

Smith, K.M., and Xeros, N. 1954b. A comparative study of different types of viruses and their capsules in the polyhedroses and granuloses of insects. Parasitology, 44, 400-406.

Smith, K.M., Trontl, Z.M., and Frist, R.H. 1964. A note on a granulosis virus disease of a Noctuid larva. Virology, 24, 508-513.

Sorvall Ivan, Inc. 1965. Thin sectioning and associated technics for electron microscopy, 2nd ed. Norwalk, Connecticut, U.S.A.

Stahler, N. 1939. A disease of the corn ear worm, Heliothis obseleta (F.). J. Econ. Entomol., 32, 151.

Stairs, G.R. 1964. Selection of a strain of insect granulosis virus producing only cubic inclusion bodies. Virology, 24, 520-521.

Stairs, G.R. 1965. Quantitative differences in susceptibility to nuclear-polyhedrosis virus among larval instars of the forest tent caterpillar, Malacosoma disstria (Hübner). J. Invertebrate Pathol., 7, 427-429.

Steinhaus, E.A. 1947. A new disease of the variegated cutworm, Peridroma margaritosa (Haw.). Science, 106, 323.

Steinhaus, E.A. 1948. Polyhedrosis ("Wilt disease") of the alfalfa caterpillar. J. Econ. entomol., 41, 859-865.

Steinhaus, E.A. 1949. "Principles of Insect Pathology". MacGraw-Hill, New York.

Steinhaus, E.A. 1951. Possible use of Bacillus thuringiensis Berliner as on aid in the biological control of the alfalfa caterpillar. Hilgardia, 20, 359-381.

Steinhaus, E.A. 1960a. The duration of viability and infectivity of certain insects pathogens. J. Insect Pathol., 2, 225-229.

Steinhaus, E.A. 1960b. Notes on polyhedroses in Peridroma, Prodenia, Colias, Heliothis, and other Lepidoptera. J. Insect Pathol., 2, 327-333.

Steinhaus, E.A., and Dineen, J.P. 1960. Observations on the role of stress in a granulosis of the variegated cutworm. J. Insect Pathol., 2, 55-65.

Steinhaus, E.A., and Marsh, G.A. 1960. Granulosis of the granulate cutworm. J. Insect Pathol., 2, 115-117.

Steinhaus, E.A., and Thompson, C.G. 1949. Preliminary field tests using a polyhedrosis virus to control the alfalfa caterpillar. J. Econ. Entomol., 42, 301-305.

Steinhaus, E.A., Hughes, K.M., and Wasser, H.B. 1949. Demonstration of the granulosis virus of the variegated cutworm. J. Bacteriol., 57, 219-224.

Stelzer, M.J. 1965. Susceptibility of the great basin tent caterpillar, Malacosoma fragile (Stretch), to a nuclear-polyhedrosis virus and Bacillus thuringiensis Berliner. J. Invertebrate Pathol., 7, 122-125.

Stern, V.M., Hall, I.M., and Peterson, G.D. 1959. The utilization of Bacillus thuringiensis Berliner as a biotic insecticide to suppress the alfalfa caterpillar. J. Insect Pathol., 1, 142-151.

Summers, M.D., and Paschke, J.D. 1968. Observations on an artifact present in Trichoplusia ni granulosis-virus preparations. J. Invertebrate Pathol., 11, 520-523.

Svensson, H., and Valmet, E. 1955. Density gradient electrophoresis - a new method of separating electrically charged compounds. Sci. Tools, 2, 11-13.

Tanada, Y. 1961. The epizootiology of virus diseases in field populations of the armyworm, Pseudaletia unipuncta (Haworth). J. Insect Pathol., 3, 310-323.

Tanada, Y. 1962. Effects of a microsporidian spore suspension on the incidence of cytoplasmic polyhedrosis in the alfalfa caterpillar, Colias eurytheme Boisduval. J. Insect Pathol., 4, 495-497.

Tanada, Y., and Chang, G.Y. 1964. Interactions of two cytoplasmic-polyhedrosis viruses in three insect species. J. Insect Pathol., 6, 500-516.

Tanada, Y., and Chang, G.Y. 1968. Resistance of the alfalfa caterpillar, Colias eurytheme, at high temperatures to a cytoplasmic-polyhedrosis virus and thermal inactivation point of the virus. J. Invertebrate Pathol., 10, 79-83.

Tanada, Y., and Leutenegger, R. 1968. Histopathology of a granulosis-virus disease of the codling moth, Corpocapsa pomonella. J. Invertebrate Pathol., 10, 39-47.

Tanada, Y., and Reiner, C. 1962. The use of pathogens in the control of the corn earworm, Heliothis zea (Boddie). J. Insect Pathol., 4, 139-154.

Tanada, Y., and Tanabe, A.M. 1964. Response of the adult of the armyworm, Pseudaletia unipuncta (Haworth), to cytoplasmic-polyhedrosis-virus infection in larval stage. J. Insect Pathol., 6, 486-490.

Tanada, Y., Tanabe, A.M., and Reiner, C.E. 1964. Survey of the presence of a cytoplasmic-polyhedrosis virus in field populations of the alfalfa caterpillar, Colias eurytheme Boisduval, in California. J. Insect Pathol., 6, 439-447.

Teakle, R.E. 1969. A nuclear-polyhedrosis virus of Anthela varia (Lepidoptera: Anthelidae). J. Invertebrate Pathol., 14, 18-27.

Thompson, C.G. 1959. Thermal inhibition of certain polyhedrosis virus diseases. J. Insect Pathol., 1, 189-190.

Thompson, C.G., and Steinhaus, E.A. 1950. Further tests using a polyhedrosis virus to control the alfalfa caterpillar. Hilgardia, 19, 411-445.

Todd, E.L. 1955. The distribution and nomenclature of the corn earworm. J. Econ. Entomol., 48, 600-603.

Tompkins, G.J., Adams, J.R., and Heimpel, A.M. 1969. Cross infection with Heliothis zea using nuclear-polyhedrosis viruses from Trichoplusia ni. J. Invertebrate Pathol., 14, 343-357.

Tripconey, D. 1969. A nuclear polyhedrosis of the lucerne caterpillar, Colias electo. J. Invertebrate Pathol., 14, 111-114.

Tripconey, D. 1970. Studies on a nonoccluded virus of the pine tree emperor moth. J. Invertebrate Pathol., 15, 268-275.

Vago, C. 1963. A new type of insect virus. J. Insect Pathol., 5, 275-276.

Vago, C., and Bergoin, M. 1968. Viruses of invertebrates. Advances Virus Res. 13, 247-303.

Vail, P.V., and Gough, D. 1970. Susceptibility of the pupal stage of the cabbage looper, Trichoplusia ni, to nuclear-polyhedrosis virus. II. Histopathology. J. Invertebrate Pathol., 15, 211-218.

Vail, P.V., and Hall, I.M. 1969. The influence of infection of nuclear-polyhedrosis virus on adult cabbage loopers and their progeny. J. Invertebrate Pathol., 13, 358-370.

Valentine, R.C. 1961. Contrast enhancement in the electron microscopy of viruses. Advances Virus Res., 8, 287-318.

Van der Geest, L.P.S. 1968. A method for the purification of polyhedra. J. Invertebrate Pathol., 11, 502.

Van Regenmortel, M.H.V. 1960. Zone electrophoresis and electron microscopy of a watermelon mosaic virus from South Africa. Virology, 12, 127-130.

Van Regenmortel, M.H.V. 1961. Zone electrophoresis and particle size of cucumber mosaic virus. Virology, 15, 221-223.

Van Regenmortel, M.H.V. 1964a. Separation of an antigenic plant protein from preparations of plant viruses. Phytopathology, 54, 282-289.

Van Regenmortel, M.H.V. 1964b. Purification of plant viruses by zone electrophoresis. Virology, 23, 495-502.

Van Regenmortel, M.H.V. 1968. Zone electrophoretic $R_{\emptyset}$ values of plant viruses. S.A. Med. J., 42, 118.

Van Regenmortel, M.H.V., and Fowle, L.G. 1962. Separation of two plant viruses by zone electrophoresis. S. African J. Agr. Sci., 5, 515-517.

Van Regenmortel, M.H.V., Nel, A.C., and Hahn, J.S. 1964. Separation of three plant viruses by zone electrophoresis. S. African J. Agr. Sci., 7, 817-822.

Van Son, G. 1949. "The Butterflies Of Southern Africa". Part 1. The Transvaal Museum, Pretoria.

Venable, J.H., and Coggeshall, R. 1965. A simplified lead citrate stain for use in electron microscopy. J. Cell Biol., 25, 407-408.

Watanabe, H. 1967. Development of resistance in the silkworm, Bombyx mori, to peroral infection of a cytoplasmic-polyhedrosis virus. J. Invertebrate Pathol., 9, 474-479.

Watson, M.L. 1955. The use of carbon films to support tissue sections for electron microscopy. J. Biophys. Biochem. Cytol., 1, 183-184.

Watson, M.L. 1958a. Staining of tissue sections for electron microscopy with heavy metals. J. Biophys. Biochem. Cytol., 4, 475-478.

Watson, M.L. 1958b. Staining of tissue sections for electron microscopy with heavy metals. II. Application of solutions containing lead and barium. J. Biophys. Biochem. Cytol., 4, 727-730.

Whitcomb, R.F., Jensen, D.D., and Richardson, J. 1968. The infection of leafhoppers by western X-disease virus. V. Properties of the infectious agent. J. Invertebrate Pathol., 12, 192-201.

Wilcox, J., Howland, A.F., and Campbell, R.E. 1956. Investigations of the tomato fruitworm, its seasonal history and methods of control. Tech. Bull., 1147. U.S.A. Dept. Agr., Washington, D.C.

Xeros, N. 1952. Cytoplasmic polyhedral virus diseases. Nature, 170, 1073.

Xeros, N. 1956. The virogenic stroma in nuclear and cytoplasmic polyhedroses. Nature, 178, 412-413.

ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr. A. Polson for his guidance and encouragement throughout all stages of this study; to Professor A. Kipps for help in the preparation of the manuscript; to Dr. D.J.M. MacKenzie, Dr. B.W. Russell and Miss L.M. Stannard for reading the thesis; to Mrs. M. Hodgkiss for help with the illustrations; and to all members of the Virus Research Unit for kind co-operation. Thanks are also due to Dr. E.D.F. Williams, for the use of the ultramicrotome and to Mr. H. Geertsema, for entomological advice.

For financial support from the Department of Agricultural Technical Services and the South African Medical Research Council I express sincere appreciation.