VIRUSES OF *HELIOTHIS ARMIGERA* (HÜBNER) (LEPIDOPTERA : NOCTUIDAE)

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ABSTRACT

Heliothis armigera (the bollworm) is a pest of agricultural importance in Southern Africa. It is often simultaneously infected with several insect viruses: nuclear polyhedrosis virus (NPV), granulosis virus (GV), and cytoplasmic polyhedrosis virus (CPV).

Both NPV and GV have been previously studied because of their ready availability and their potential use in pest control. However, there have been no recorded studies of CPV in *H. armigera* because of the small amounts of virus present in the naturally infected larvae. Particular emphasis was therefore placed in this study on the CPV of *H. armigera*.

CPV was successfully separated from naturally occurring NPV by a combination of techniques, such as differential centrifugation, density gradient zone electrophoresis and absorption with antibody to NPV. The CPV was then successfully propagated by passage in larvae reared on synthetic media. Pure CPV having been obtained, its replication and physico-chemical properties could be studied. Serological studies using immune electron microscopy and immune osmophoresis were used to detect and identify viruses of *H. armigera* and examine relationships between different CPVs.

The gross morphological appearance of viral infections in *H. armigera* were studied. Distinctive features were observed both in larvae infected by NPV and in those infected by GV, but CPV-infected larvae were not markedly different in appearance from uninfected larvae. Larvae died readily from NPV or GV infection indicating their suitability for use in controlling *H. armigera*, but there was no larval mortality following CPV infection.

Histopathological examination of infected larval tissues and organs in the midgut further demonstrated the characteristics of each virus infection. It was shown that NPV and GV infect cell nuclei, predominantly those of fat body cells; most tissues of the larvae were infected by NPV, whereas CPV replicated and was assembled only in the cytoplasm of the midgut cells. Electron microscopy showed that the rod-like morphology of NPV and GV differed from the icosahedral CPV particle. A further distinguishing feature was the arrangement into inclusion bodies and the inclusion body structure of each virus type.

Several physico-chemical properties of the virions were determined. Buoyant densities, determined isopycnically in CsCl gradients, yielded values of 1.444 g cm^{-3} and 1.242 g cm^{-3} for CPV and NPV respectively. Sedimentation coefficients of 399 S and 1188 S were found for CPV and NPV respectively. A diffusion coefficient of 0.73×10^{-7} cm^2s^{-1} was determined for CPV using the quantitative gel precipitin technique, and a partial specific volume of 0.69 g ml^{-1} was obtained by sedimentation velocity in water and heavy water. Hence the molecular weight of CPV of *H. armigera* could be estimated, and this was confirmed by the methods of particle counting and sedimentation equilibrium. An average value of 47.4×10^6 was calculated. The diameter of the CPV particle, measured from electron micrographs, was 59 nm. The NPV virions were 368 by 89 nm.

The nucleic acids of all three virus types were characterized, greater emphasis being placed on that of CPV. Those of NPV and GV were shown to be double-stranded (ds) DNA, while that of CPV was ds RNA. The latter consisted of ten well-separated genome segments when analysed by polyacrylamide gel electrophoresis (PAGE). This facilitated the determination of their molecular weights. The relationship of log molecular weight to electrophoretic mobility for ds RNA species on PAGE was shown to be a curve, rather than a straight line, as previously thought. On the basis of this curve, the molecular weights of the genome segments of the CPV RNA were determined by comparative studies with reovirus type 3 and using ^{32}p-labelled RNA. The total molecular weight of the genome segments was 12.37×10^6.

An interesting feature of the CPV RNA was a change in the polyacrylamide gel electrophoretic pattern of the genome segments in CPV passaged in the laboratory compared with CPV found in the natural state. The change consisted in the disappearance of a high-molecular-weight genome segment and the appearance of a fast-moving segment of low molecular weight - a reproducible phenomenon observed with several isolates from the field. The field isolate was named CPV_{A1} to distinguish it from the virus grown in the laboratory, CPV_{A2}. The total molecular weight of the nucleic acid of CPV_{A1} was estimated to be 14.27×10^6, as opposed to 12.37×10^6 for CPV_{A2}.

A different CPV was isolated from fresh supplies of *H. armigera* obtained for rearing purposes. This virus (CPV_B) had genome segments that migrated on PAGE in the same manner as that of a CPV from *Nudaurelia cytherea cytherea,* which was also isolated in this study. The molecular weight of its RNA genome was found to be 14.02×10^6, by comparative PAGE with CPV_{A2} of *H. armigera.*

The protein molecular weights were determined by comparative PAGE. The dissociated CPV virions were found to consist of proteins ranging in molecular weight from approximately 13 400 to 163 000. The molecular weight of the major protein was approximately 32 400. Dissociated polyhedra contained proteins ranging in molecular weight from 11 800 to 145 000. The molecular weight of the major polyhedral protein of CPV and NPV was approximately 28 000. This protein stained positively with Schiff's reagent indicating that it contained carbohydrate.

Attempts to grow NPV either in bacteria or yeasts were unsuccessful. No replication was observed within the microorganisms. The mortality of larvae fed a suspension of NPV incubated with microorganisms was no greater than larvae fed the original NPV inoculum.

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INTRODUCTION

This thesis describes some of the viruses that infect *Heliothis armigera*. With the present awareness of environmental pollution, insect viruses are likely to play an increasing role as alternatives to chemical insecticides in combating insect pests. Part of the present work is, therefore, directed towards a detailed study of viral infection in *H. armigera*.

At least three viruses are known to infect *Heliothis:* nuclear polyhedrosis virus (NPV), granulosis virus (GV), and cytoplasmic polyhedrosis virus (CPV). Previous studies have been concerned with GV and NPV (Gitay, 1971; Whitlock, 1974), which are of direct agricultural importance. The large yields from larvae and the ease of isolating these viruses have facilitated their study, whereas little attention has been directed towards CPV, which is more difficult to isolate and purify (Smith, 1967).

There has been some confusion in the past regarding viruses isolated in the laboratory, because it has not always been clear whether the extracted virus was the one used for inoculation or a naturally occurring latent virus. It is therefore imperative that viruses reported should be characterized as fully as possible. Further confusion has been evident in the different systems of classification that have been described (Lwoff and Tournier, 1971). In the past, the criteria for classification included host range, cytopathology and site of viral replication. The more recent classification devised by the International Committee on Virus Nomenclature (ICNV) (Wildy, 1971) makes extensive use of biochemical and biophysical characteristics, and includes the invertebrate viruses within a unified system of viruses.

As there is little information available on the CPV of *H. armigera*, it was selected as the major topic for this study. This thesis seeks to characterize the virus biophysically and biochemically, to elucidate its biological role, and to compare it serologically with other available CPVs and other viruses of *H. armigera*. Although the CPV viruses isolated in this study are morphologically similar, serological techniques have shown antigenic differences between them.

The cytoplasmic polyhedrosis viruses are members of the Reoviridae, which are of particular interest since they are widely distributed in nature: reovirus and bluetongue viruses infect mammals; the wound tumour virus (WTV) infects plants and the CPV infects insects (Wildy, 1971; Joklik, 1974). These viruses are similar in their structural, physical and chemical properties, despite different host specificities (Wood, 1973), but CPV is characterized by the occlusion of particles within crystalline inclusion bodies. In addition, the Reoviridae have a unique divided double-stranded RNA genome, which is of importance for molecular biological studies. The similarity between the CPVs and vertebrate reoviruses emphasizes the need to establish parameters by which viruses may be precisely identified and compared.

Since it was found during this study that NPV and GV are often closely associated with CPV, some of their characteristics are also described. A major problem was the low concentration of CPV in field isolates. Techniques were devised to provide CPV in quantities suitable for use in a wide range of investigations.

Following a report by Wells (1970) describing the propagation of insect viruses in microorganisms, attempts were made to duplicate these findings in this laboratory. These experiments were unsuccessful and are summarized in Appendix 1.

The first two chapters of this thesis review the relevant literature and describe the host insects and materials. The third is devoted to the methods employed to separate and isolate the viruses, with special reference to CPV. This is followed in Chapters 4 and 5 by a study of the serological relationships and the symptomatology of NPV, GV, and CPV infections in *H. armigera*. Chapter 6 presents a histopathological study of the different viral infections. The following three chapters (7-9) describe the characterization of the viruses by a variety of physical and chemical techniques. The implications of the results are discussed at the end of each chapter as well as in Chapter 10.

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CHAPTER 1

REVIEW OF THE LITERATURE

The literature review will confine itself to those areas of insect virology and biophysics relevant to this study. It is presented in the order of topics dealt with in this thesis.

1.1 INSECT VIRUSES

Insects act either as vectors for animal and plant viruses or as hosts for their specific viruses (Smith, 1967). The economic importance of insect viruses is two-fold. On the one hand, viruses may cause disease as for example in silkworms, and their replication may thereby jeopardize the sericulture industry. On the other hand, replication of certain viruses may be encouraged in organized programmes of biological control of insect pests that devastate important agricultural crops; such programmes need not markedly alter the ecosystem. The specificity of insect viruses for this purpose should be carefully determined, and it is essential to fully characterize the viruses. The potential ecological hazards when a replicating virus is released into the ecosystem have been stressed by Tinsley and Melnick (1973/4).

There are two main groups of insect viruses: the occluded and the nonoccluded. In the first group, the virions are occluded within protein 'crystals' (polyhedron, capsule or spherule), whereas in the second group, the viruses are nonoccluded.

1.1.1 Occluded Viruses

Members of this group are infectious when administered *per os* to insects (Bergold, 1947; Smith and Xeros, 1952). The occluded viruses are subdivided on the basis of their morphology, their site of replication and their biochemical and biophysical properties (Wildy, 1971).

Baculoviridae

This group includes the nuclear polyhedrosis viruses (NPVs) and granulosis viruses (GVs). Both types of virion are rod-shaped, and are enveloped in a protein 'crystal'. The NPVs produce polyhedral inclusion bodies (Bergold, 1947) and the virions are arranged within polyhedra, either as singly enveloped virions, or as bundles of nucleocapsids, common to a single envelope. The GV is usually ovi-cylindrical and the inclusion bodies are termed capsules. One or two virions are enclosed within the capsules (Paillot, 1926; Steinhaus, 1947; Tanada, 1959). Baculoviruses contain double-stranded DNA (Wyatt, 1952; Summers and Anderson, 1972a) and grow within the nuclei of infected cells (Smith and Xeros, 1953a).

Cytoplasmic Polyhedrosis Viruses

The cytoplasmic polyhedrosis viruses (CPVs) are characterized by the production of polyhedra in the cytoplasm of infected cells (Smith and Wyckoff, 1950), usually in the epithelial cells of the midgut. The polyhedra contain icosahedral virus particles whose genome has been characterized as double-stranded (ds) RNA (Miura *et al.*, 1968; Fujii-Kawata *et al.*, 1970). The CPVs have been described in over eighty species of insects (Smith, 1963) and have recently been included as a genus within the Reoviridae (Joklik, 1974). They do, however, differ from reoviruses by their host range, morphology of virions and the production of polyhedra.

Insectpox Viruses

Viruses resembling the animal pox viruses, e.g. vaccinia, are occluded within spherical 'crystalline' bodies (spherules) in the cytoplasm of the fat body cells (Granados and Roberts, 1970). This group was named Entomopoxvirus, subgenus of Poxvirus, by the International Committee of Nomenclature of Viruses (Wildy, 1971) and is included in the family Poxviridae (Melnick, 1975). Virus particles contain ds DNA.

1.1.2 Nonoccluded Viruses

These form a heterogenous group including spherical, or polyhedral, or rod-shaped viruses which are not surrounded by a protein matrix but are free in insect tissues. Sites of replication are either the nucleus or the cytoplasm. Work is in progress in order to recognize types among nonoccluded RNA viruses (Wildy, 1971).

1.2 HOST INSECTS USED IN THIS STUDY

1.2.1 *Heliothis armigera*

Heliothis armigera belongs to the order Lepidoptera and family Noctuidae. It was first described by Fabricius in 1793 as *Bombyx obsoleta*. After considering the nomenclature and synonymy, Nel (1958) concluded that the correct name of this moth is *Heliothis armigera* (Hübn.). *Heliothis armigera*, commonly known as the American bollworm, is of economic importance in South Africa where it attacks a variety of crops. A detailed list of host plants includes cotton, tomatoes, maize, citrus, deciduous fruits, tobacco and leguminous plants such as the pea, lupin and lucerne (Nel, 1958).

Life-cycle and Description of *Heliothis armigera*

The stages in the life-cycle have been described by Nel (1958) and Whitlock (1972). The egg is almost spherical and measures 0.48 × 0.5 mm. If fertile, it is pearly white and darkens just before hatching, whereas infertile eggs are yellow and remain so. In the field, the incubation period of the egg is 3 to 5 days during the summer, and 9 days in winter. Freshly hatched larvae are 1.5 mm long. They are whitish grey with a dark head. Older larvae vary in colour from grey to pink and green, and have a dark dorsal longitudinal band extending from behind the head to the ninth abdominal segment. The larvae progress through five instars, reaching an average length of 40 mm and average mass of 390 mg at maturity.

Pupation occurs in the soil and, depending on weather conditions, the emergence from the egg follows in 14 to 33 days. The pupae are yellowish-green just after pupation, changing to reddish-brown prior to adult eclosure. The average pupal length is 19 mm. The genital opening of the female is situated ventrally on the eighth abdominal segment and that of the male, ventrally on the ninth abdominal segment. The pupae remain in the soil for about 13 to 18 days before the adult emerges. The moths are usually nocturnal, although oviposition can occur during the day (Nel, 1958). Moth body length ranges from 18 to 22 mm and wing span from 35 to 40 mm. The front wing is light olive-green or red-brown with a pale grey undersurface, and black marking on the subterminal margin of the wing. The hind wing is similarly coloured with black marking near the edge of the wing. The male can be distinguished from the female by the head colour, which in the male is olive-green, whereas in the female it is red-brown. Another distinguishing feature is that the male is smaller than the female (Fig. 2.1). The average life-span is nine days in the male and seventeen days in the female, during which period more than 1000 eggs may be laid (Nel, 1958).

The difficulties usually encountered in maintaining a continuous supply of *H. armigera* arise from the cannibalistic tendencies of these larvae, their susceptibility to disease, the erratic mating of adults and the production of infertile eggs (Nel, 1958). A variety of cages have been used in the past to breed the moths. A wire cage was used by Ellisor (1935), but wire mesh tested by Nel (1958) was found to cause wing damage. Nel (1958) therefore used an oviposition cage consisting of two wire hoops fitted with a muslin cylinder. He suspended the cage which contains one male and one female 1.05 m above the ground. A cage made from perspex and nylon mesh was specially devised for conditions in the present study (see Section 2.3) and was successfully used to breed *H. armigera*.

Viruses infecting *H. armigera*

The first reference to a naturally occurring insect virus disease in *Heliothis* was made by Mally (1891) quoted by Ignoffo (1973) and known as 'caterpillar wilt' disease. Further observations on *H. obtectus* were made by Mally (1905) reported by Lounsbury (1913). Chapman and Glaser (1915) also reported a 'wilt disease' in *H. obsoleta*. The responsible virus agent was demonstrated by Parsons only in 1936 and later confirmed by several workers, including Stahler (1939), Steinhaus (1949) and Smith (1963).

Viruses reported to infect *H. armigera* under natural conditions are the nuclear polyhedrosis virus (NPV) (Bergold and Ripper, 1957); cytoplasmic polyhedrosis virus (CPV) (Rubinstein *et al.*, 1975) and granulosis virus (GV) (Gitay and Polson, 1971). The comparative symptomatology of both the NPV and GV affecting *H. armigera* was studied by Whitlock (1974). Single infections may occur, but more often larvae are infected with two or all three of these viruses. Infections of both NPV and CPV in *Heliothis* were reported by Smith and Rivers (1956) and confirmed by Steinhaus (1960). Dual infection of *H. armigera* with NPV and GV was noted by Whitlock (1974) and multiple infections have also been observed in other Lepidoptera (Smith and Xeros, 1953b; Bird, 1966; Smith, 1967). All three groups of occluded viruses were found in *H. armigera* during the present study.

1.2.2 *Nudaurelia cytherea cytherea* (Fabr.)

Nudaurelia cytherea cytherea (Fabricius), the pine tree emperor moth, is a Lepidopterous species belonging to the family Saturniidae (Fig. 2.2). It occurs in the broad coastal belt of the Cape Province and on the slopes of the adjoining mountain range (Geertsema and Giliomee, 1972). The larvae feed on a variety of food plants including sugarbush, eucalyptus and fruit trees, but show a marked preference for the exotic pine, *Pinus radiata* D. Don. *N. cytherea cytherea* is a serious forest pest at times causing extensive defoliation and stunting of tree growth (Geertsema and van den Berg, 1973).

The earliest report of the occurrence of *N. cytherea cytherea* in Fort Cunyghame State Forest (Eastern Cape) and the effects of a fungal disease upon its larvae was by Sims (1903). A polyhedral wilt disease of this insect was reported by Tooke and Hubbard (1941) and a nonoccluded virus was first isolated from it by Hendry *et al.* (1968). These larvae are susceptible to virus infection which causes disease and high mortality. The first symptoms are loss of appetite and mobility, followed by flaccidity and liquefaction of larval organs. Infected larvae may reach pupation before dying (Tripconey, 1970).

Five viruses have been isolated from *N. cytherea cytherea* (Juckes, 1970); these viruses were differentiated and identified as the α, β, γ, δ and ε particles. The β particle had been the most intensively studied, as it usually appears in large quantities, is easily extractable, and is thought to be highly pathogenic, causing drastic reductions in the numbers of this pest (Hendry *et al.*, 1968; Tripconey, 1970).

In the present study attention was focused on the α particle because it appeared to be a CPV (Juckes, 1974) and could thus be used in an important comparative study with the CPV of *H. armigera.*

1.3 FEATURES OF VIRAL INFECTION IN INSECTS

Insect viruses usually attack the larval stage of Lepidoptera for the adult insect shows greater resistance to infection. The signs of infection will vary depending on the tissues affected and thus on the nature of the particular virus. Detailed descriptions of the symptoms of insect virus diseases have been reviewed by Smith (1967).

The first general symptom of NPV infection is cessation of feeding by the larvae. The skin, haemocytes, fat body, trachea and silk glands are major sites of infection resulting in the typically 'oily' appearance of the skin which may rupture, liberating the liquefied body-contents as infection progresses. Death follows in 4 to 21 days. In the silkworm, *Bombyx mori*, the skin becomes yellow in patches accounting for the term 'jaundice' first used to describe the illness. The NPV-infected larvae typically move to the highest available position either on the plant or in the laboratory container, where they suspend themselves by the pro-legs.

The symptomatology of *H. armigera* infected with NPV was first described by Lounsbury (1913) in conjunction with Mally in observations on field trials conducted in lucerne fields.

A colour change, usually a lightening in colour, is a prominent feature of GV-infected larvae (Smith, 1967). The main site of viral replication is fat body tissue and after death, a cream-like liquid containing GV capsules is extruded.

The appearance of CPV-infected larvae differs from larvae infected with baculoviruses in that the site of CPV viral replication is predominantly in the midgut (Smith, 1967). Associated with CPV infection is reduced feeding, impaired reproduction and decreased larval, pupal and moth size (Neilson, 1965; Ignoffo and Adams, 1966). Both the development and fecundity of *Pectinophora gossypiella* are affected by CPV (Bullock *et al.*, 1970). Adults which develop from CPV-infected larvae of the cabbage looper were found to be smaller than control adults (Vail and Gough, 1970). Similarly, reduction in pupal weight, adult emergence and egg production in CPV-infected *H. virescens* has been noted (Simmons and Sikorowski, 1973), and in *Porthetria dispar* by Magnoler (1974) who found, in addition, the presence of structural abnormalities and melanotic inclusions, especially in diseased females.

Bioassays are essential before field trials can be undertaken to determine effectiveness of entomogenous viruses. Bioassays may be used:

(1) to examine the host range of a virus;

(2) to determine the relative biological activity of viruses against one or more hosts;

(3) to ascertain the relative infectivity and virulence of different preparations of the same agent; and

(4) as an analytical tool to isolate and characterize the agent (Vail, 1975).

Bioassays by injection of inclusion bodies into larvae in the laboratory are not sufficiently accurate. An alternative method is one in which known quantities of inclusion bodies are included in, or applied to, the surface of the diet of the larva, so that mortality may be recorded at specified time intervals. This method (Ignoffo, 1966) was used in the present study.

The first record of the use of NPV to control *H. armigera* was by Coaker in 1958. McKinley (1971) successfully infected *H. armigera* with NPV in the laboratory but field tests in cotton plantations in Rhodesia were not as effective. Studies were also reported for *H. armigera* infected with NPV in the Ivory Coast (Angelini and Labonne, 1970), in Botswana (Roome, 1971; Daoust, 1974; Daoust and Roome, 1974) both for a field and laboratory assay. In a comparative study, Roome (1975) tested NPV of *H. armigera*, commercial preparations of *H. zea* NPV, and *Bacillus thuringiensis* on *H. armigera* in the field, and found that the local NPV *H. armigera* virus was the most effective. The results of these investigations deserve to be confirmed in order to indicate future lines of biological pest control in the field.

1.3.1 Host Specificity and Latency of Viral Infection

A large number of insect species have been found to be susceptible to infection by two or more viruses. These infections may occur simultaneously (Bird, 1966; Smith and Xeros, 1953b). Viruses may interact by interference, synergistic association or mutual co-existence (Tanada and Chang, 1964). Aruga *et al.* (1961) and Aruga *et al.* (1963) were the first to observe interference in a study on two CPV viruses of *B. mori*.

Insect viruses were thought to be host specific but several studies have described successful transmission of insect viruses between host species (Bergold, 1958; Aizawa, 1963; Smith, 1963). Specificity of insect viruses has ben reviewed by Ignoffo (1968). In his review he stated that insect viruses are mainly specific to the class Insecta, GV being the most specific.

Studies by Cunningham (1968) and Cunningham and Longworth (1968) have shown that NPV from four species of insects and CPV from seven species respectively were serologically indistinguishable, which may indicate that many viruses isolated from different hosts and known by the specific name of the host may be closely similar, if not identical.

Opinions have differed with respect to the existence of latent viruses. Aruga (1963) suggested that activation of latent viruses rather than true cross-transmission occurs. Examples of latent virus infections have been described by Smith and Xeros (1953) in their work on *Vanessa cardui*, in which they thought that an NPV and CPV were being transmitted. However, in a subsequent publication (Smith and Rivers, 1956), activation of a latent virus was thought to have occurred.

Smith (1967) suggested that transovarial transmission occurs either by contamination of the egg surface or within the egg, thus accounting for latent viruses. For CPV, transovarial transmission seems likely, as most evidence points to the fact that larvae, on emerging from the egg, become infected by virus acquired from the surface of the egg. This was confirmed by Mery and Dulmage (1975) who prevented the transmission of CPV from one generation to another in *H. virescens* by treating the egg with 0.2% solution of sodium hypochlorite for 8 min. They therefore suggested that the virus was transmitted on the surface, rather than within the egg.

1.4 PROPERTIES OF INCLUSION BODIES

1.4.1 Morphology of Polyhedra

The term polyhedron, as defined by Steinhaus and Martignoni (1970), is a crystal-like inclusion body which encloses a number of polyhedrosis-virus particles and is produced in the cells of tissues affected by certain insect viruses. Because of the presence of mature virus particles within inclusion bodies of insect viruses, these inclusion bodies differ from those present in other infections.

The size and shape of polyhedra associated with different viruses differ greatly (Hukuhara and Hashimoto, 1966). Gershenson (1959, 1960) and Aruga *et al.* (1961) considered that the polyhedral shape is virus specific, rather than host specific. This was confirmed by Hukuhara and Hashimoto (1966) who described hexahedral and icosahedral-shaped polyhedra produced by two CPVs in the same host. The hexahedral polyhedron was a regular cube and the icosahedron seemed to be hexagonal in outline when viewed in the optical microscope. A detailed study of the polyhedral forms was made with the aid of a scanning electron microscope by Rao (1973). He described 12-faced bodies, corresponding to the icosahedron reported by Hukuhara and Hashimoto (1966), as a rhombic dodecahedron. The range in size and shape of CPV polyhedra is more extensive than that found in NPV polyhedra. Despite variation in adjacent cells, Tanada (1960) found that each cell has polyhedra of similar size. Aruga and Israngkul (1961) considered that the size of polyhedra depended on the time between infection and examination of the polyhedra. This observation was supported in the present study, as it was found that there was a progressive increase in size of polyhedra obtained from CPV-infected *H. armigera*. Variations in the size of polyhedra have been reported by Hukuhara and Hashimoto (1966) and Stairs *et al.* (1968).

1.4.2 Purification of Polyhedra

To isolate occluded viruses it is necessary first to purify the inclusion bodies and then to liberate viral particles from them.

The development of methods for purifying polyhedra was facilitated by observations showing that they were insoluble in water (Bolle, 1873 *in* Bergold, 1963a), resistant both to putrefaction (Jacques and Huston, 1969) and to treatment with organic solvents such as fluorocarbon (Bergold, 1959). Baculovirus inclusion bodies are more easily purified than those of CPV, as the latter may break up when kept for long periods in water (Wittig *et al.*, 1960).

Additional purification of polyhedra was achieved using sucrose solutions or density-gradient centrifugation. Van der Geest (1968) purified large amounts of homogenized polyhedra by centrifugation through a 61.7% (*m/m*) sucrose solution followed by centrifugation through a 43.9% (*m/m*) sucrose solution. Khosaka *et al.* (1971) centrifuged polyhedra through

50% sucrose to remove contaminating material.

Isopycnic separation of NPV was successfully accomplished by centrifugation in sucrose density-gradient columns in swinging-bucket rotors (Martignoni, 1967) and in zonal rotors (Martignoni *et al.*, 1968). In the latter study, one of the few studies which measured the reduction in bacterial contamination, the total bacterial flora was considerably reduced after purification of the polyhedra. Hayashi and Bird (1970) treated partially purified suspensions of polyhedra with 1% sodium deoxycholate, digested with a mixture of ribonuclease A and trypsin, and centrifuged the resulting mixture over a discontinuous gradient consisting of 1.5 M sucrose layered over 2.2 M sucrose. This technique is the basis of the method most frequently used in the present study for purifying inclusion bodies.

Martignoni (1967) developed an isopycnic method to purify and separate CPV and NPV inclusion bodies by making use of the small differences in their respective densities. Preliminary experiments in the present study which attempted to separate CPV and NPV polyhedra by means of this technique were unsuccessful. Tompkins and Adams (1970) had also attempted to use this technique to separate two types of polyhedra, but were unsuccessful and were only able to separate released virions.

1.4.3 Digestion of Polyhedra

The extraction and partial purification of baculoviruses from their inclusion bodies was first described by Bergold (1947), who dissolved NPV polyhedra in 0.03 M Na_2CO_3 and 0.05 M NaCl. Since that time the use of specific concentrations of alkali for inclusion bodies from different viruses, has become the basis of most methods of digestion. Hukuhara and

Hashimoto (1966) found that a carbonate buffer caused less damage to the virus particles than the buffer used by Bergold (1947). The liberation of virus particles from CPV polyhedra is more difficult because CPV viruses disintegrate easily in weak alkali (Smith, 1967). An effective method is that of Hills and Smith (1959) in which a polyhedral suspension is dialyzed against a weak Na_2CO_3 solution followed by dialysis against distilled water. Virions prepared by treating polyhedra with dilute carbonate solutions, were shown to be less infective when used for cell culture than free virus particles (Vago and Bergoin, 1963).

Polyhedra and capsule inclusion bodies are disrupted in the gut of insects resulting in the release of virions. Polyhedra have been considered as having a framework of silicates (Estes and Faust, 1966) which are solubilized by alkaline chloride solutions. It has been suggested that gut juices solubilize polyhedra because of the high pH acting in conjunction with enzymes. Vago and Croissant (1959) studied the action of gut juice from *Bombyx mori* on NPV polyhedra. When polyhedra were treated with undiluted gut juice partial breakdown of polyhedra and partial release of intact virus particles occurred in 100 to 130 seconds. After 60 minutes of exposure to gut juice, many of the rods released were fragmented. Both Aizawa (1962) and Aruga and Watanabe (1964) noted the antiviral activity of gut juice, but this was unlikely to be due to digestive enzymes as Faust and Adams (1966) found that dissolution of polyhedra proceeded after gut fluid had been heated at 100^0C to inactivate any enzymes. Hayashi and Bird (1970) observed that enzymes such as RNase, DNase, trypsin and chymotrypsin had no effect on viral structure but removed impurities adsorbed to the virion surface. With the preliminary isolation of a trypsin-like enzyme from the midguts of *H. armigera*, attention was focused, in the present study, on the application of trypsin digestion for inclusion bodies.

1.6 SEROLOGICAL STUDIES

With the re-evaluation of insect viruses to control insect pests, the use of serological techniques to identify and monitor insect viruses in the field is of increasing importance.

Serological techniques have been used to investigate relationships between inclusion bodies and inclusion body proteins. Tanada (1954) used agglutination and precipitation techniques to show that serologically related inclusion bodies were closely associated with the host species, rather than with the type of inclusion body formed by the virus. In addition, Tanada (1954) studied the relationship between inclusion bodies and inclusion body proteins. From his results he concluded that the NPV of *Pieris rapae* and *Colias eurytheme* were closely related.

In studies on the serological relationship of inclusion body proteins of NPV and GV, Krywienczyk and Bergold (1960a, 1961) found a cross-reaction between *Porthetria dispar* and *Lymantria monacha*, and cross reactivity between the capsule proteins of *Recurvaria milleri* with the lepidopteran polyhedral proteins. In a further study on the serological properties of inclusion body proteins of *Pieris brassicae* GV, Longworth *et al.* (1972) found two distinct proteins. The first was a protein typical of inclusion body proteins and the second a protein present at the surface of the enveloped virus particle and in the inclusion body envelope.

A comparative serological study was undertaken by Payne (1971) of the polyhedral proteins of CPV from *Nymphalis io*, *Aglais urticae*, *Arctia caja*, *Phalera bucephala* and *B. mori*. He observed cross-reactions between all of these CPVs with the exception of *B. mori* CPV.

Serological techniques have also been used to study the relationship between inclusion body protein and virus particles. Such studies were undertaken by Bergold (1947), Krywienczyk and Bergold (1960b) and Shapiro and Ignoffo (1970). A positive serological relationship was reported by Shapiro and Ignoffo (1970) for the viral and polyhedral proteins of *Heliothis zea* NPV. Krywienczyk and Bergold (1960c) had suggested that positive cross-reactions in early studies may have occurred because of inadequate purification of virus particles as any remaining polyhedral protein would stimulate antibody formation.

Finally, these techniques have also been used to demonstrate a serological relationship between virus particles from a variety of hosts. Early attempts to produce antibody to CPV by Neilson (1964) did not succeed, the presumed reason being the destruction of viral antigenicity by the procedures used to liberate the viral particles. A different technique was therefore used by Hukuhara and Hashimoto (1966) to isolate CPV and they were able to demonstrate that the free virions from the icosahedron and hexahedron viruses of *B. mori* were closely related. They also showed that CPV particles from both the hexahedral and icosahedral polyhedra of *B. mori* were unrelated to the NPV of *B. mori*. Following this work, Cunningham and Longworth (1968) used complement fixation to study the serological relationships between several CPV viruses. These were from insects of families Nymphalidae, Arctiidae, Hepialidae, Lasiocampidae and Notodontidae. They appeared to be serologically related, except for the CPV virus from *B. mori*. Subsequently Krywienczyk *et al.* (1969) tested highly purified CPV viruses by double diffusion and immunoelectrophoresis, and found that viruses from *Malacosoma disstria* and *Orygia leucostigma* shared five antigens in contrast with only one or two with the CPV from *B. mori*.

Ikegami and Francki (1973) showed that antibodies present in an antiserum produced against a virus containing double-stranded (ds) RNA also reacted with ds RNA from other sources. Therefore serological cross-reactions observed between ds RNA viruses may result from antibody-ds RNA complexes, rather than from more specific virus-antibody reactions. This finding was confirmed by Payne and Kalmakoff (1974). However, at high dilutions of antiserum (1/1280) the cross-reaction with ds RNA disappeared, whereas the virus-specific reaction still occurred. Apart from serological relationships, it is also possible to detect differences between ds RNA virus isolates by study of their nucleic acid profiles. This technique was used in the present study.

Techniques commonly used to detect serological relationships include agar gel diffusion (Krywienczyk and Bergold, 1961); diffusion on cellulose acetate (Krywienczyk and Cunningham, 1970); agglutination and precipitation (Tanada, 1954); complement fixation (Hukuhara and Hashimoto, 1966; Cunningham and Longworth, 1968) and haemagglutination which was used by Cunningham *et al.* (1966). Several of these techniques were tested but were found unsuitable for the present study. However, lesser used techniques in the study of insect viruses, such as immune electron microscopy and immune osmophoresis, were found to give useful information and are therefore briefly reviewed below.

1.6.1 Immune Electron Microscopy

Immune electron microscopy may be used not only to visualize antigen-antibody reactions, but, by complexing virus particles with their homologous antisera, can also be used to identify viruses occurring in very low concentrations.

Early attempts to visualize virus-antibody complexes using electron microscopy were only partially successful in that, although clumping of particles was observed, the antibody molecules could not be seen. Anderson and Stanley (1941) were the first to observe a reaction between tobacco mosaic virus (TMV) and homologous antiserum, whereas no reaction was noticed between the particles and control serum. Shadow casting was used by Williams and Wyckoff (1945) and Easty and Mercer (1958) to obtain three-dimensional studies of antigen-antibody complexes. However, it was only after the introduction of negative staining by Brenner and Horne (1959) that Anderson *et al.* (1961) was able to demonstrate its use in examining antibody-antigen complexes. This technique was further developed in studies on influenza virus by Lafferty and Oertelis (1963), who, after careful preparation of their material, found evidence for the bivalent attachment of one molecule of antibody to two antigenic sites on the same virus. Similar procedures were used by Almeida *et al.* (1963) in their work on human wart and polyoma virus. Subsequent studies on influenza virus particles by Brown and Smale (1970), in which IgG and IgM were used and antiserum absorbed with subviral particles, indicated three distinct antigenic sites.

These developments led to the successful application of immune electron microscopy to insect viruses in a study of the β particles of *N. cytherea cytherea* by Marais (1970).

A recent review by Kapikian (1975) describes an application of the method to detect and identify agents of human disease by using the patient's serum as the antibody source.

1.6.2 Immune Osmophoresis

Immune osmophoresis is also known as counter immuno-electrophoresis or counter-electrophoresis. Bussard and Huet (1959) reported this technique as a combination of electrophoresis and immunological precipitation enhanced by electro-endosmosis. The term immuno-osmophoresis was first used by Bon and Swanborn (1963) because the movement of antibody was due to electro-endosmotic flow. In a subsequent study on plant viruses, Ragetli and Weintraub (1964) noted that the technique allowed rapid identification and quantitative evaluation of high-molecular weight antigens and in addition reported that it was a hundred times more sensitive than agar-gel diffusion for TMV. This method was used by Alter *et al.* (1971), White *et al.* (1971) and Kenny *et al.* (1972) for sensitive and specific detection of pneumonoccal polysaccharide. While the present study was in progress, use of this technique was reported in a study of the serological relationships of twelve small RNA insect viruses (Reinganum and Scotti, 1976).

1.7 HISTOPATHOLOGICAL STUDIES

Studies on the replication of insect viruses have been mainly undertaken in whole insects. Sites of viral replication have been determined by histological examination and by the study of thin sections using electron microscopy. These techniques demonstrate which tissues are infected by the different types of occluded viruses, NPV, CPV, and GV, after the ingestion of food contaminated with these viruses.

Studies on NPV of Lepidoptera, for example *Pectinophera gossypiella* and *Trichoplusia ni*, show that it multiplies predominantly in nuclei of fat body cells, hypodermis, tracheal matrix, and nerve tissue (Vail and Jay, 1973; Hunter *et al.*, 1973). Other tissues infected include muscle, malpighian tubules, haemocytes, ganglia, and pericardial cells. In some species, infection of the midgut has been reported (Heimpel and Adams, 1966; Young *et al.*, 1972 and Vail and Jay, 1973), whereas this was rarely seen by Mathad *et al.* (1968), and usually only when the surrounding connective tissue was heavily infected.

In a comparative study of NPV and GV infections, Hamm (1968) showed that GV attacked only the fat body cells causing proliferation of the cells, whereas NPV infected a wide variety of tissues with no resulting cell proliferation. In contrast, the studies on GV by Huger and Krieg (1961) and Tanada and Leutenegger (1970) revealed virus particles and virogenic stroma in midgut epithelial cells, but encapsulated virus was seen mainly in fat cells.

The ultrastructure of baculovirus-infected cells has been studied by several workers, including Smith and Xeros (1953a), Bird (1964), Kislev *et al.* (1969), Summers and Arnott (1969), and Hunter *et al.* (1973). A detailed study of the initial stages of GV infection was undertaken by Summers (1969, 1971). After being released from the capsular protein, the virion becomes attached to membranes of the gut columnar epithelium and then loses its membrane during entry into the cell. A similar process of entry was described for NPV by Harrap (1970).

Summers (1971) found no evidence of phagocytosis, whereas ultrastructural studies of NPV in *B. mori* cells *in vitro* revealed entry of the membrane-enclosed virion into the cell by phagocytosis or viropexis (Raghow and Grace, 1974). Attachment of the particle to the nuclear membrane at the nuclear pore appears to be the next stage enabling the entry of viral DNA into the nucleus (Summers, 1971; Raghow and Grace, 1974). Raghow and Grace (1974) also showed that viral DNA synthesis and virion assembly took place after the appearance of electron-dense material, and that naked viral rods appeared (90% becoming membrane-enclosed), and were occluded within polyhedra. In contrast with other studies (Heimpel and Adams, 1966) showing that polyhedra contain either singly enveloped virions or bundles of nucleocapsids, Raghow and Grace (1974) observed both structural arrangements within the same polyhedra. Despite previous conflicting reports, Harrap (1972a) confirmed the existence of a polyhedron membrane.

Studies on the ultrastructure of baculoviruses showed that the rod-shaped particles are enclosed in inner and outer membranes, also referred to as intimate and developmental membranes (Bergold, 1963). The existence of two membranes surrounding the virions has been confirmed by Bergold (1963a). More detailed studies by Harrap (1972b) on NPV demonstrated that the virus envelope consisted of three layers, whereas the inner membrane was composed of subunits arranged in a loose lattice. Beaton and Filshie (1976) recently compared the ultrastructure of GV and NPV using optical diffraction, their results indicating that the capsids of the two viruses were indistinguishable. Their recommended terminology of 'capsid' in the place of 'intimate membrane', and 'virus membrane' instead of 'developmental membrane', is in line with current terminology for animal and plant viruses and will be used in the present study.

Harpaz *et al.* (1965) studied the histological changes in the midguts of CPV-infected larvae, and like Xeros (1956), noted changes only in the columnar cells of the epithelium. Further studies by Xeros (1966) revealed, in addition, virogenesis in goblet cells. These observations were verified by Stairs *et al.* (1968), who found that most cells of the larval midgut of *Estigmene acrea* were susceptible to a CPV. According to Martignoni *et al.* (1969) polyhedra in columnar and goblet cells are arranged in apicobasically oriented rows.

Ultrastructural studies of CPV development showed that it matures only in the cytoplasm, without in the initial stages of infection, affecting the nucleus (Bird, 1966). The dense 'virogenic stromata', consisting of protein and RNA (Xeros, 1956), included virus particles during their maturation in the cytoplasm (Stoltz and Hilsenhoff, 1969). The virus particles developed from the stromata before polyhedral formation and particle occlusion (Xeros, 1966). During polyhedral formation, virogenic stroma increased in size and showed an absence of normal cytoplasmic components (Stoltz and Hilsenhoff, 1969). The virogenic stroma found early during the infection was followed by the 'crystallogenic matrix', which Arnott *et al.* (1968) distinguished from the virogenic stroma in work on CPV infection of *Danaus plexippus*. The presence of free virus particles (i.e., not occluded in polyhedra) was observed by Stairs *et al.* (1968) and later confirmed by Hayashi (1970), who observed three times as many free as occluded particles. These observations were also made in the present study.

In an unusual strain of CPV of *Bombyx mori*, which forms inclusions predominantly in the nuclei of midgut cells, the virus particles were present only in the cytoplasmic inclusions, but not in nuclear inclusions (Hukuhara and Yamaguchi, 1973).

1.7.1 Structure of CPV virions

Occluded and nonoccluded CPV virions have the same morphology (Hayashi and Bird, 1968). The icosahedral shape of the particle was demonstrated electron microscopically by Hosaka and Aizawa (1964), who used shadowing techniques. The existence of cores has been reported by Bergold and Suter (1959) and Wittig *et al.* (1960), and verified by Hosaka and Aizawa (1964) and Lewandowski and Traynor (1972), who showed that the particles consisted of an electron-dense core surrounded by an electron-transparent membrane or shell. Other studies of the ultrastructure of CPV particles have shown that they consist of two concentric icosahedral shells (Arnott *et al.*, 1968; Hosaka and Aizawa, 1964), each shell having twelve subunits localized at twelve vertices of the icosahedron (Miura *et al.*, 1969) which are associated with the five-fold axis of symmetry (Hosaka and Aizawa, 1964). A structure similar to the double shell of CPV appears to correspond to the inner layer observed in reovirus particles (Vasquez and Tournier, 1964).

The segmented hollow projections thought to protrude from the subunits of the outer shell (Hosaka and Aizawa, 1964) were described by Lewandowski and Traynor (1972) as originating from the central core of the virus. Hosaka and Aizawa (1964) described four segments, and Arnott *et al.* (1968), only two. The projections are 20 nm long (Miura *et al.* 1969), with spherical structures (12 nm in diameter) at their tips; these structures were described by Asai *et al.* (1972) and were also observed in the present study.

1.8 PHYSICO-CHEMICAL PROPERTIES

1.8.1 Biophysical studies

In the past, studies on the properties of insect viruses have been mainly concerned with identification of the host and biological specificity for the host. Relatively few studies have used physico-chemical measurements to characterize the virus, but recently increasing interest has been shown in the definition of the nucleic acids and proteins of insect viruses as an aid to classification (Payne and Rivers, 1976).

Previous studies on the viruses of *H. armigera* (Gitay, 1971; Whitlock, 1974) did not undertake a detailed examination of the viruses in terms of their physico-chemical properties, hence particular attention was given to them in this study. Juckes (1974) characterized the β and ε viruses of *N. cytherea cytherea* in considerable detail since they were obtained in large quantities, but adequate amounts of the α (or CPV) particle could not be obtained. Measurements of the properties of this virus described in the present study were achieved following the development of suitable concentration and purification techniques.

Reports of the properties of virus particles are often inconsistent, in part owing to their instability in different buffer systems, and also to the different purification procedures applied to them. A further point of confusion has been that different laboratories, working with viruses of the same name, may in fact have been working with different viruses.

Important features for accurate identification of viruses are particle size and density, sedimentation coefficient, diffusion constant, particle molecular weight and nucleic acid and protein composition.

There is considerable variation in the size measurements of CPV particles, most values lying between 40-70 nm (Table 1.1), whereas baculoviruses are less variable in their size range. The buoyant density is a property which varies with the material used to make the gradient and often bears little relation to the true density of the particle (Gerin *et al.*, 1968; Rowlands *et al.*, 1971). Buoyant densities have been determined mainly for the CPV of *B. mori* by Lewandowski and Traynor (1972); Payne and Kalmakoff (1974). A comparison of values reported in the literature, together with other physical properties of CPV viruses, is listed in Table 1.1.

The variation in sedimentation coefficients calculated for different viruses may either signify differences between virus particles or may indicate inadequate purification before sedimentation. From a knowledge of the sedimentation coefficient, diffusion constant and the partial specific volume, molecular weight values may be determined. A common difficulty in this method which makes use of the Svedberg equation (Section 7) is the accurate estimation of the diffusion coefficients of viruses.

Experimental methods to measure the diffusion coefficient (*D*) were reviewed by Gosting (1956) and by Markham (1967). For large macromolecules having slow diffusion, Dubin *et al.* (1967) described a method using light scattering, but although this gives accurate results, it has the disadvantage of requiring specialized equipment and relatively large amounts of material. Because of the small amounts of virus available in the present study, an alternative immuno-chemical procedure, developed by Polson (1958), and based on quantitative gel precipitin tests, was used. This method is a refinement of the double-diffusion tube method of Oakley and Fulthorpe (1953) in which the distance from the antigen meniscus to the precipitin band in columns of gel was shown to be a linear function of the negative logarithm of the antigen concentration. From this, *D* could be calculated (see Section 7.4).

The estimation of molecular weight, using either Svedberg's equation (combination of *s* and *D*) or equilibrium centrifugation, requires a knowledge of the partial specific volume ($\bar{\nu}$). For purified protein preparations, a value for $\bar{\nu}$ can be calculated from amino acid composition, but for viruses it is not accurate (Bancroft and Freifelder, 1970). Sedimentation equilibrium experiments in water and heavy water to measure molecular weight and $\bar{\nu}$ were reviewed by Edelstein and Schachman (1973). Sedimentation velocity methods in aqueous and heavy water solutions require small samples and are less subject to errors than most other techniques. It was used by Martin *et al.* (1956) to determine the $\bar{\nu}$ of bovine serum albumin, sodium alginate and polyvinyl alcohol, and their results were comparable with those obtained by other methods. In a further study using the same technique, Martin *et al.* (1959), determined the $\bar{\nu}$ of several macromolecules, and in addition studied changes due to isotopic exchange. They showed their measurements of $\bar{\nu}$ to be accurate, and expressed the extent of isotopic exchange as a factor κ which they found was 1.0150 in pure D_2O for many proteins.

For viruses, as opposed to proteins, sedimentation velocity in

D_2O was first used by Sharp et al. (1950), who showed that D_2O had little effect on influenza virus. Bellamy *et al.* (1974) determined the partial specific volume of two oncornaviruses by sedimentation in mixtures of D_2O and H_2O containing 5mM Tris and 1mM EDTA pH 7.0. Concentrated virus was suspended in various mixtures of buffered D_2O-H_2O and allowed to stand at 0^0C for 5 h to permit D_2O-H_2O exchange to take place. The virus was concentrated by ultracentrifugation and resuspended in the appropriate D_2O-H_2O mixture to determine the sedimentation coefficient by analytical ultracentrifugation. This method is the basis of that used to determine $\bar{\nu}$ for CPV of *H. armigera*.

Molecular weights of viruses have frequently been derived by combination of sedimentation and diffusion coefficients, but the most versatile and accurate method is that of sedimentation equilibrium (Van Holde, 1967). A widely used technique is the 'high-speed method' of Yphantis (1960) in which the ultracentrifuge is run at a sufficiently high speed so that the concentration of the solute becomes essentially zero at the meniscus; the interference fringe count through the cell image becomes a direct measure of the absolute concentration of solute at any position in the cell. Yphantis (1964) used a rotor speed of 2378 rpm (close to the lower limit then available) to determine a value of 6.5×10^6 for the molecular weight of southern bean mosaic virus. Bancroft and Freifelder (1970) determined the molecular weights of coliphages by 'high-speed' equilibrium centrifugation using the lower limit of 800 rpm. They obtained a molecular weight of 49×10^6 for T_7 phage that was within the range expected for CPV of *H. armigera*. This method was used in the present study for the molecular weight of CPV of *H. armigera*.

A comparison of the reported range of values for the physical properties of some Reoviridae and Baculoviruses is listed in Table 1.1.

TABLE 1.1 REPORTED PROPERTIES OF SOME REPRESENTATIVE REOVIRIDAE

Virus	Size (nm)	Density in CsCl (g/cm^{-3})	Sedimentation coefficient-s S	Particle Weight (x 10^6)	%RNA	Number of genome segments	RNA (x 10^{-6})	Protein (x 10^{-4})
CPV								
Bombyx mori	63^{a}		400^{f}		23^{j***}	9	$15-2^{c*}$	
	69^{b}	1.435^{b}	440^{e}	54^{s}			12.7^{e}	52^{b}
	60^{c}		430^{d}	2.87^{c*}		9	12.81^{j**}	47^{h}
	62^{d}	1.37^{g}		97^{c}			$14-18^{g}$	
		$1.43-1.48^{h}$				10	14.6^{r}	
Nymphalis io	48^{i}					10	14.4^{h*}	
Arctia caja	52^{i}		376^{i}					
	50^{i}	1.45^{h**}						
Malacosoma disstria			380^{j*}			16	20.77^{j***}	
Euproctis chrysorrhoea			373^{i}					
Orygia leucostigma			380^{m}		free virions 28^{j} occluded 26^{j}			
Phalera bucephala	55^{i}							
Aglais urticae	50^{i}		391^{i}					
Pectinophora gossypiella	35.7^{k}							
Pseudaletia unipuncta	$49.3-106.9^{l}$ mean 75.6							
33 CPV isolates, 11 distinct types						10	$13.62-15.53^{h***}$	
Reovirus	65^{n}	$1.36-1.38^{n*}$	630^{n*}		14.6^{n*}		15^{x}	88^{y}
	70^{o}	1.44^{p} cores	470^{q}					
Wound tumour virus	60^{u} $66^{b,r}$		514^{s}		23^{s}		15^{x}	50^{y}
Blue Tongue virus (L)	$69^{s,t*}$	$1.36-1.38^{s}$	550^{s}		20^{t}		12^{t}	48^{y}
(D)	$60^{s,t*}$	$1.38-1.42^{t}$	$470^{s,t*}$		30^{s}		12^{s}	28^{y}
Rice Dwarf virus	70^{v}		512^{w}		12^{y}		15.3^{r}	110^{y}

SOME REPRESENTATIVE BACULOVIRIDAE

							DNA (x 10^{-6})	
NPV								
Trichoplusia ni	300 x $72^{3\dagger}$		1228^{3}					
	300 x $33^{\dagger\dagger}$		1530^{3}					
" tissue culture		1.26-1.35^{4}	825^{4}					
" after sodium deoxycholate treatment		1.42^{4}	325^{4}					
H. armigera	320 x $90^{1\dagger}$							
H. zea	336 x 62^{10}						40-50^{9}	
Ceramica picta	312 x 40^{2}							
Bombyx mori	330 x $80^{12\dagger}$						2^{7}	
	360 x $60^{12\dagger\dagger}$							
Porthetria dispar	300-330 x 100-140^{11}†							
	300-320 x 40-50^{11}††							
Aglais urticae	260-300 x 60-70^{11}†							
	200-250 x 40-50^{11}††							
GV								
Choristoneura murinana			1324^{5}	460				
Pseudaletia unipuncta (Haworth)	411 x 62^{13}							
T.ni.							100^{6}	
Spodoptera frugiperda							95^{6}	
Agrotis segetum							30^{8}	

† = with envelopes

†† = without envelopes

REFERENCES

I - Reoviridae

a - Hosaka and Aizawa (1964)
b - Lewandowski and Traynor (1972)
c - Miura *et al.* (1969)
c^* - Miura *et al.* (1968)
d - Kawase *et al.* (1973)
e - Kalmakoff *et al.* (1969)
f - Hukuhara and Hashimoto (1966)
g - Nishimura and Hosaka (1969)
h - Payne and Kalmakoff (1974)
h^* - Payne and Tinsley (1974)
h^{**} - Payne (1976)
h^{***} - Payne and Rivers (1976)
i - Cunningham and Longworth (1968)
j - Hayashi and Bird (1968)
j^* - Hayashi and Bird (1970)
j^{**} - Hayashi and Krywienczk (1972)
k - Ignoffo and Adams (1966)
l - Tanada and Chang (1960)
m - Richards and Hayashi (1970)
n - Gomatos *et al.* (1962)
n^* - Gomatos and Tam (1963)
o - Loh and Shatkin (1968)
p - Skehel and Joklik (1969)
q - Zweerink *in* Joklik (1974)
r - Fujii-Kawata *et al.* (1970)
s - Martin and Zweerink (1972)
t - Verwoerd (1969)
t^* - Verwoerd *et al.* (1972)
u - Bils and Hall (1962)
v - Fukushi and Shikata (1963)
w - Suzuki and Kimura (1969) (cited by Joklik, 1974)
x - Reddy and Black (1973b)
y - Joklik (1974)

II - Baculoviruses

1 - Bergold and Ripper (1954)
2 - Adams *et al.* (1968)
3 - Scott *et al.* (1971)
4 - Henderson *et al.* (1974)
5 - Bergold (1958)
6 - Summers and Anderson (1972b)
7 - Onodera *et al.* (1965)
8 - Shvedchikova and Tarasevich (1971)
9 - Scharnhorst *et al.* (1977)
10 - Gregory *et al.* (1969)
11 - Harrap (1972b)
12 - Khosaka *et al.* (1971)
13 - Tanada, (1959)

1.8.2 Nucleic Acid Studies

The demonstration by Avery *et al.* (1944) that bacterial transformation was effected by DNA and the proposal by Hershey and Chase (1952) that viral nucleic acid molecules carry information for progeny virus was confirmed with the finding that tobacco mosaic virus RNA was itself infectious (Gierer and Schramm, 1956). Subsequently much work has centred on the nature and structure of viral nucleic acids.

Classification of viruses and the understanding of virus replication depends largely on detailed knowledge of nucleic acid structure (Wildy, 1971). Thus nucleic acids of any new virus must be characterized, particularly with respect to conformation and size. Viruses contain only one of the two types of nucleic acid, DNA or RNA, whose configurations can vary widely. All the following configurations have been described in virions:

DNA (i) single-stranded (ss) linear
(ii) ss circular
(iii) double-stranded (ds) linear
(iv) ds circular

RNA (i) ss, some with segmented genomes and
(ii) ds, most with segmented genomes

Gomatos *et al.* (1962) were the first to show that reovirus-infected cells contained RNase-resistant material. Montagnier and Sanders (1963) detected RNA intermediates with a double-stranded structure in Krebs II mouse ascites cells infected with encephalomyocarditis virus. At the same time, Gomatos and Tamm (1963) showed that RNA from reovirus virions had physico-chemical properties typical of ds RNA. Some of these characteristics are:-

a sharp melting profile with a melting temperature (Tm) measured at 50% level of hyperchromicity dependent on ionic concentration;

resistance to ribonuclease digestion;

and a base composition with approximately equimolar amounts of adenine (A) and uridine (U), and of guanine (G) and cytosine (C).

Langridge and Gomatos (1963) provided further evidence of the ds nature of RNA by X-ray diffraction studies which showed a typical double-helical structure.

The International Committee for the Nomenclature of Viruses (ICNV) recently proposed that all 'reovirus-like' viruses be placed into the Reoviridae family (Joklik, 1974). The criteria for classification are based on morphology, genome size and structure. The genomes must consist of about 10-12 molecules of ds RNA with a combined molecular weight of 15×10^6 daltons. Among viruses containing ds RNA included in this family are several plant, insect and animal viruses:

(i) The genus *Orthoreovirus* (reovirus) in which the host range is confined to vertebrates includes the three morphologically identical serotypes of reovirus (Shatkin *et al.*,1968).

(ii) The genus *Orbivirus*, in which the host range includes insects and vertebrates. The most extensively studied example is bluetongue virus (BTV) (Verwoerd, 1969).

(iii) A genus whose host range includes plants and insects. Well-known members of this group are wound tumour virus (WTV) (Kalmakoff *et al.*,1969; Reddy *et al.*,1975), rice dwarf virus (RDV) (Miura *et al.*,1966), maize rough dwarf virus (MRDV) (Harpaz, 1972; Reddy *et al.*,1975), and Fijii disease virus (FDV) (Teakle and Steindl, 1969).

(iv) A genus comprising the CPVs whose host range is limited to insects.

The nucleic acids of CPV have been studied mostly in the CPVs of *B. mori* (Nishimura and Hosaka, 1969; Fujii-Kawata *et al.*,1970; Lewandowski and Millward, 1971; Hayashi and Krywienczyk, 1972). The nucleic acid genome of CPVs of *Malacosoma disstria* (Hayashi and Krywienczyk, 1972) and *Nymphalis io* (Payne and Tinsley, 1974) have also been studied. Recently Payne and Rivers (1976) compared the nucleic acids of thirty-three isolates of CPV and found eleven distinct CPV types according to their profiles on polyacrylamide gel electrophoresis.

Double-stranded RNA forms exist as intermediates in viral replication (for example, in polio ds RNA (Baltimore, 1964), whether as a fully ds replicative form of unknown function or as the true replicative intermediate with ss tails.

A number of physico-chemical procedures can be used to characterize nucleic acids:

> Colorimetric reactions (Dische, 1930, cited by Burton, 1956) are more or less specific for pentose or deoxyribose sugars, and distinguish RNA from DNA.
>
> Determination of buoyant densities, also distinguish between RNA and DNA, and, in addition may help to distinguish between single and double strandedness.
>
> Thermal denaturation studies can show whether the nucleic acid is single or double-stranded, and enzyme digestion can provide information regarding its nature and structure.

Further characterization of nucleic acids can be achieved using polyacrylamide gel electrophoresis (PAGE), a technique originally developed to separate proteins (Raymond and Weintraub, 1959; Reisfield *et al.*, 1962). It has since been applied to the separation of ss RNA species (Richards *et al.*, 1965; Loening, 1967, 1969; Bishop *et al.*, 1967) and to the resolution of ds RNA species (Shatkin *et al.*, 1968). In addition, DNA fragments obtained by digestion with restriction endonucleases have been separated, and supercoiled and nicked open circular ds DNA species are well separated both from each other and from high molecular weight linear DNA (Harley and White, 1973).

A segmented genome appears to be characteristic of nearly all ds viral RNAs, but the size distribution is specific for each virus. The molecular weights of the genome segments are therefore an important parameter for characterizing the genetic material. The classical methods for determining absolute molecular weight frequently require large quantities of material and show poor resolution of segmented genomes, especially when genomes are similar in size. Relative methods are more frequently used. Electron microscopy, though potentially absolute, requires internal standards because of the elasticity of the molecules. Although accurate, it cannot resolve groups of molecules with similar molecular weights. Sucrose gradient zonal sedimentation (Britten and Roberts, 1960) is frequently used but suffers from the same disadvantage. Only PAGE can successfully resolve genome segments with small differences in molecular weights.

Bishop *et al.* (1967) calibrated a system in which they used PAGE to separate ten different viral nucleic acid species. A linear relationship between log molecular weight and relative electrophoretic mobility was shown for ss RNA of molecular weights ranging from 3×10^5 to 2.3×10^6. However, the conformational state of RNA affects its mobility in PAGE. To overcome this, the method was later modified by using gels polymerized in denaturing solvents such as formaldehyde (Boedtker, 1971) and formamide (Staynov *et al.*,1972). Subsequently Wellauer and Daiwid (1973) showed that formaldehyde treatment did not eliminate secondary structure in HeLa cell rRNA.

Other variables that could account for errors in determining molecular weight are temperature during electrophoresis (Groot *et al.*, 1970; Grival *et al.*,1971), the base composition of the RNA under study, variation in ionic strength of the electrophoresis buffers (Loening, 1969) and the acrylamide concentration (Fisher and Dingman, 1971). The electrophoretic mobility varies with gel concentration for a given species, and is characteristic of its conformational properties, differing for ss, ds, linear, and circular species (Harley *et al.*,1973; Harley and White, 1973).

Reovirus RNA segments have been found to fall into three size-groups; designated large, medium and small (L, M and S) on the basis of both rate-zonal centrifugation and electron microscopic studies (Bellamy *et al.*,1967; Dunnebacke and Kleinschmidt, 1967) with molecular weights corresponding to 2.5, 1.4, and 0.8 x 10^6 daltons, respectively. Using PAGE, these groups were further resolved into 3 L, 3 M, and 4 S species for reovirus (Shatkin *et al.*,1968). It was assumed that the same relationship between log molecular weight and electrophoretic mobility held for ds RNA as for ss RNA. Shatkin *et al.* (1968) based their assumption on a plot of the average distance migrated on PAGE for each of the three classes of segments, versus their average molecular weight determined by sedimentation analysis. The graph plotted showed the best straight line drawn through only three data points. The size of individual genome fragments was then calculated from their respective electrophoretic mobilities by reference to this linear plot.

Although no definitive study to confirm the linearity of this relationship has been reported, comparative PAGE has become the standard method used to estimate molecular weights of ds RNA, based on reovirus standards (Fuijii-Kawata *et al.*, 1970; Lewandowski and Traynor, 1972; Wood, 1973; Sekiguchi and Koide, 1974; Reddy *et al.*, 1974; Payne and Tinsley, 1974).

High-molecular-weight ds linear DNA migrates as a single band on PAGE (Harley and White, 1973) although rate-zonal sedimentation shows it to be heterogeneous in molecular size. With increasing molecular weight, the electrophoretic mobility approaches a lower limiting value at any fixed gel concentration. This is contrary to the concept of a linear relationship between log molecular weight and electrophoretic mobility for high-molecular-weight ds DNA. Supportive evidence for doubting the validity of a linear relationship for ds RNA is that the electrophoretic mobility characteristics of ds RNA in PAGE have been shown to be generally similar to those of ds linear DNA (Harley *et al.*,1973).

Other studies have questioned the validity of the PAGE comparative method for molecular weight determination. Molecular weights obtained by centrifugation and gel electrophoretic methods often vary widely (Grivell *et al.*,1971; Reijnders *et al.*,1974), and the use of different PAGE markers as standards may also influence the results (Marchoux *et al.*,1973). It would be necessary to elucidate these problems before definitive molecular weight measurements can be made.

1.8.3 Molecular weights of viral proteins

Viral protein characterization includes the determination of protein molecular weight, which is necessary for identification and for providing comparisons with other viruses.

Direct measurement of protein molecular weights using physical methods such as equilibrium sedimentation are often unsatisfactory because viral proteins tend to aggregate (Durham, 1972). This tendency may be overcome by the use of detergents such as SDS, which are frequently used in conjunction with mercaptoethanol, urea, or acetic acid. Proteins are thereby separated into individual soluble polypeptide chains, which can then be fractionated on the basis of size on acrylamide gels containing SDS. The comparative method of PAGE represents a rapid and convenient method for obtaining the approximate molecular weights of viral proteins. It requires only small amounts of protein and measures molecular weights relative to known standards.

Ornstein and Davis first described disc electrophoresis in 1959 (in Maurer, 1971) but the theoretical and practical aspects were developed subsequently. Shapiro *et al.* (1967) incorporated SDS into the solvent system, finding an inverse linear relationship between relative migration distance and log molecular weight. As markers, they used eleven well-characterized proteins, which they denatured and reduced in 1% SDS and 1% 2-mercaptoethanol. They showed that the relationship between relative migration and log molecular weight was linear for molecular weights $15 - 170 \times 10^3$ but was hyperbolic for the entire range tested.

This study was extended by Weber and Osborn (1969), who tested the reliability of the technique with forty well-characterized proteins. They concluded that molecular weights could be determined with an accuracy better than ± 10%, and also showed that electrophoretic mobility was independent of the isoelectric point and amino acid composition, and seemed governed solely by the molecular weight of the polypeptide chains.

Polyacrylamide pore gradients may be used to enhance the separation of different-sized proteins (Slater, 1965). The migration rate of proteins in linear gradient gels decreases progressively until each protein reaches the 'pore' limit (Slater, 1968), producing a band of increased sharpness.

Many variations of SDS-PAGE have been developed for viral proteins since Maizel (1966) separated the polypeptides of adenovirus. Both viral structural and non-structural proteins have been determined (Maizel, 1971).

Early studies on the proteins of insect viruses were restricted to analyses of the amino acid composition. A report of the amino acid content of the NPV of *B. mori* was made by Desnuelle and Chang (1943). A later study of the amino acid composition of the NPV and CPV icosahedral and hexahedral strains of *B. mori* showed that the composition of the polyhedra was similar (Kawase, 1964).

Lewandowski and Traynor (1972) using the PAGE technique studied the polypeptide composition of CPV particles from *B. mori* by dissociating the particles with urea-SDS and labelling them with ^{125}I. Five virion polypeptides were revealed with molecular weights of approximately 151 000; 142 000; 130 000; 67 000 and 33 000.

The polyhedral protein comprised two major polypeptides with approximate molecular weights of 29 000 and 19 500 and three minor polypeptides with molecular weights of 122 000; 100 000 and 48 000. For reovirus, the RNA segments appear to code for proteins which are similar in size to the theoretical primary gene products from each segment (Smith *et al.*,1969). This may equally apply to the CPVs. Lewandowski and Traynor (1972) showed that the five CPV polypeptide species could be coded by five of the monocistronic mRNA species while the polyhedral polypeptides could be direct gene products of the five remaining genome cistrons. Similar results were obtained by Payne and Tinsley (1974) and Payne and Kalmakoff (1975). In the latter study on *B. mori* CPV, five polypeptides were observed for CPV virions and five for polyhedra; however the molecular weights obtained for some of the species differed from those found by Lewandoski and Traynor (1972). A study on the structural proteins of CPV of *Nymphalis io* (Payne and Tinsley, 1974) revealed three polypeptides in the virions with molecular weights ranging from 116 000 - 30 000. The major polypeptide of polyhedral protein had a molecular weight of 37 000 and appeared to be a glycoprotein.

In a comprehensive study in which both the RNA and virus structural proteins of thirty-three isolates of CPVs were analysed, Payne and Rivers

(1976) used the RNA genome profiles on PAGE in conjunction with data on the viral proteins as a basis for a provisional classification of various CPVs.

There have been diverse reports on the number of proteins present in polyhedra. Bergold (1947) used sedimentation values to calculate molecular weights of 15 360 and 18 270 for the polyhedral protein of NPV of *Lymantria dispar* and *L. monacha* obtained by solution of polyhedra in 0.1 - 0.5 M NaOH. Kozlov *et al.* (1969), cited by Kozlov *et al.* (1975), found a minimal subunit of *B. mori* NPV polyhedral protein dissolved in 0.1 M NaOH, to be about 11 000 daltons. In a later study using PAGE, Kozlov *et al.* (1975) found one component with a molecular weight of approximately 28 000 when polyhedra were dissolved in 67% CH_3COOH; however, when the polyhedra were dissolved in 0.05 M Na_2CO_3 and 0.1 M NaCl, pH 11.0, cleavage of the proteins occurred, and several components were revealed.

The protein composition of NPV particles appears to be more complex than that of CPV particles. Padhi *et al.* (1974) studied the proteins of NPV of *Porthetria dispar* and showed that the virus particles contained fourteen proteins ranging in molecular weights from 12 - 140 x 10^3 daltons. They found that polyhedral protein obtained by alkali treatment consisted of three components with molecular weights of 11 000, 18 000 and 29 000.

1.9 BIOLOGICAL CONTROL OF INSECT PESTS

In biological control of insect pests, the insect population may be restricted by its natural predators or by chemical insecticides. A number of pest control methods used previously for plant protection are now not as effective because certain insect populations have become resistant to some insecticides. In addition, a number of insecticides have been withdrawn because of problems associated with the accumulation of toxic residues, or are no longer available because of changed attitudes regarding environmental pollution (Smith, 1975).

Insect viruses, especially NPV and GV, have characteristics which make them ideal for use in control programs. The viruses are occluded within protective proteinaceous inclusion bodies, and their specificity and virulence avoid the broad-scale disruption of arthropod communities which results from the use of many broad-spectrum chemical insecticides. The World Health Organization (W.H.O. Tech. Report, 1973) compiled a list of 261 insects and mites of agricultural importance from which viruses have been isolated. To date, only a small number of these have been fully characterized and tested for their suitability as biological control agents.

One of the major considerations in the use of insect viruses for pest control is their general safety and non-infectivity for higher forms of life. The viruses in the W.H.O. report that have been investigated, have been subjected to exhaustive tests to determine their non-pathogenicity to man, other vertebrates and plants. Before insect viruses are used for pest control, it is of course imperative that they are accurately identified, assayed and characterized.

Studies conducted to ensure that the insect viruses are not pathogenic in warm-blooded vertebrates include attempts to grow CPV or polyhedrosis viruses in fertile chicken eggs (Steinhaus, 1951); inoculation of NPV and CPV inclusion bodies into turkey eggs (Cantwell *et al.*,1968); and attempts to passage NPV of *Heliothis* in four primate cell types obtained from human primary embryonic kidney, human diploid embryonic lung, human carcinoma of the cervix and African green monkey kidney (Ignoffo and

Rafajko, 1972).

None of these experiments could show any proliferation of the insect viruses. However, there have been several reports in the literature showing that insect viruses could multiply to a limited degree in vertebrate cell lines. Himeno *et al.* (1967) reported the production of inclusion bodies in a human amnion cell line inoculated with nucleic acid extracted from NPV of *B. mori*. McIntosh and Maramorosch (1973) were able to recover NPV viruses from vertebrate cell lines a few weeks after inoculation, while McIntosh and Kimura (1974) showed an increase in titre of the *Chilo* iridiscent virus in a vertebrate poikilothermic 'C-type' carrying cell line, four days after infection.

The production of large quantities of viruses and inclusion bodies is essential in the use of insect viruses for biological control of pests. This has generally been achieved by extracting infected material from diseased insects collected in the fields or from insects reared and infected in the laboratory (Ignoffo, 1973).

In an attempt to standardize production, several other methods of producing virus have been reported. Tissue culture methods have been generally unsuccessful for large-scale production since high yields have not, as yet, been achieved (Vago and Bergoin, 1963; **Quiot** *et al.*,1970; Ignoffo *et al.*,1971; Faulkner and Henderson, 1972). Replication of animal and plant viruses in bacterial cells and protoplasts using nucleic acids as the infective material has been successful (Bayreuther and Romig, 1964; Babbar *et al.*,1966).

The replication of insect viruses in microorganisms was described in a patent by Wells (1970) and in a subsequent publication by Wells and Heimpel (1970). The patent describes the following procedures: the microorganisms (bacteria and yeasts) were grown in nutrient broth, harvested during the late exponential growth phase and transferred to a 'transforming medium'. This medium was designed to induce structural changes in the cell wall to allow contact between virus particles and the cytoplasmic membranes. Occluded viral particles were liberated from their inclusion bodies by treatment with alkaline solution and incubated together with the microorganism. Electron and light microscopy was used to examine infected microorganisms. The growth of virus in the cells was measured by adding the infected microorganisms to media on which larvae were placed and by observing the lethal effects on them. However, in a subsequent paper by Wells and Heimpel (1970) in which only bacteria were used, no polyhedra were revealed. It is therefore unclear whether replication had, in fact, occurred.

Several attempts were made in this laboratory to reproduce these results, using both bacteria and yeasts. A variety of methods to prepare the microorganisms for infection with NPV of *H. armigera* proved unsuccessful (Rubinstein and Polson, 1976). These results are described in Appendix 1.

CHAPTER 2

MATERIALS

2.1 BUFFERS

Preparation of the buffers used throughout this work is given in the order in which they appear. Buffers were sterilized by autoclaving at 10 lbs/in^2 for 15 min unless otherwise stated.

2.1.1 Phosphate Buffer pH 7.0

(a) Disodium hydrogen phosphate anhydrous (Na_2HPO_4) 0.133 M

Na_2HPO_4	19.0 g
Distilled water	to 1 litre

(b) Potassium dihydrogen ortho phosphate (KH_2PO_4) 0.133 M

KH_2PO_4	18.25 g
Distilled water	to 1 litre

Stock solutions (a) and (b) are diluted for use as follows:

Na_2HPO_4 0.133 M	3 volumes
KH_2PO_4 0.133 M	2 volumes
Distilled water	5 volumes

The final molarity is 0.067 and pH,7.0.

2.1.2 Normal Saline

NaCl	8.5 g
Distilled water	to 1 litre

2.1.3 Borate Buffer pH 8.6

Stock solution consisted of:

H_3BO_3	62 g
NaOH	20 g
Distilled water	to 5 litre

Double-strength buffer was prepared as follows:

Borate stock	350 ml
0.1 M HCl	150 ml
Normal saline	500 ml

Single-strength buffer was prepared by addition of 800 ml distilled water to 800 ml double-strength buffer

The final concentrations were:

0.035 M H_3BO_3
0.0175 M NaOH
0.0075 M HCl
0.073 M NaCl

2.1.4 Phosphate buffered saline pH 7.0

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
Distilled water	to 800 ml

2.1.5 Barbital-glycine-tris Buffer pH 8.6

(a)

Barbital sodium	65 g
Barbital	10.35 g
Distilled water	to 5 litre

The barbital is first dissolved in a small volume of boiling distilled water.

(b) Glycine	281 g
Tris	226 g
Distilled water	to 5 litre

Equal volumes of (a) and (b) were mixed and 0.02% sodium azide added. The pH ranged from 8.5 - 8.7 with an ionic strength (*I*) of 0.08. The stock solution was diluted 1 : 3 with distilled water for use *I* = 0.02.

2.1.6 Phosphate Buffer 0.15 M pH 7.3 for Glutaraldehyde Solution

Sodium phosphate (dibasic) 0.2 M	8.1 ml
Sodium phosphate (monobasic) 0.2 M	1.9 ml

2.1.7 Veronal-acetate Buffer 0.28 M pH 7.4

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

2.1.8 Tris-HCl Buffer pH 8.0

Tris (hydroxymethyl)amino methane	1.2114 g
Distilled water	to 900 ml

Adjust pH to 8.0 with 1 M HCl.

Distilled water for final volume to 1 litre.

2.1.9 Phosphate-KCl Buffers pH 7.0

(a) Phosphate buffer pH 7.0 double-diluted with distilled water with added KCl to a final concentration of 0.1 M KCl.
I = 0.1

(b) Phosphate buffer pH 7.0 with added KCl to a final concentration of 0.2 M KCl.
I = 0.3

2.1.10 Buffers for extraction of nucleic acids:

Buffer A, pH 5.2

NaCl	0.05 M
EDTA	0.0001 M
Sodium acetate	0.1 M

Buffer B

As buffer A with SDS 1% (*m*/w) and bentonite, 0.1% (*m*/v)

2.1.11 SSC (Standard Saline Citrate)

NaCl	0.15 M
Trisodium citrate	0.015 M

The buffer is standard, i.e. 1 × SSC.

2.1.12 Buffer for Polyacrylamide Gel Electrophoresis of Nucleic Acids

Tris	0.03 M
NaH_2PO_4	0.03 M
EDTA	0.001 M
Glycerol	10% (v/v)
SDS	0.5% (*m*/v) added only when gels were not stained.

Buffers for Polyacrylamide Gel Electrophoresis of Proteins:

2.1.13 Buffer A - Tris HCl pH 8.8, 0.75 M

2.1.14 Buffer B - Tris HCl pH 6.8, 0.25 M

332 ml of buffer A to 1 litre with distilled water.
The pH is adjusted by addition of HCl.

2.1.15 Electrophoresis buffer - Tris HCl pH 8.3

Tris-HCl 0.025 M containing:

0.2 M glycine
0.1% SDS.

2.1.16 Sample buffer

Buffer B	2 ml
Glycerol	2 ml
SDS 10%	2 ml
2-Mercaptoethanol	0.2 ml

Total volume to 10 ml with distilled water.

Bromophenol Blue ...... 0.05% added as marker

For urea buffers, 4.8 g urea/10 ml buffer is added in addition or instead of mercaptoethanol.

2.2 VIRUS ISOLATES

2.2.1 Source of *Heliothis armigera*

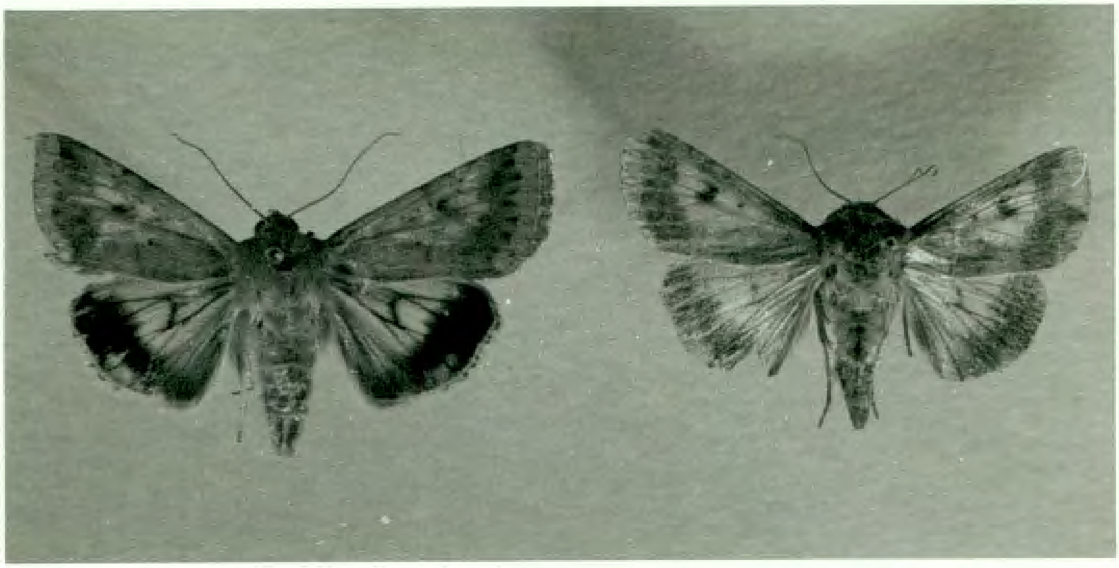
Viral material was first obtained from the Plant Protection Research Institute (PPRI) in 1972 in the form of diseased and dead *H. armigera* larvae collected in lucerne fields in the Stellenbosch district. Additional infected larvae were reared and infected at the PPRI, but an examination of the cadavers showed that the larvae from both sources contained mixtures of NPV and CPV, and, on occasion, also GV in varying proportions.

Subsequently, larvae infected only with NPV were obtained from the Department of Entomology, University of Stellenbosch. Infected larvae collected from tomato and maize crops in Rosebank, Cape, and from lupin fields in the Malmesbury-Darling districts, Cape, in 1975 and 1976 contained both NPV and CPV or CPV alone. The CPV used in this study had initially to be isolated from the original material obtained from PPRI.

Following this isolation of CPV from mixed infection of NPV and

Fig. 2.1 Adult form of *H. armigera*. The male moth is on the left, the female on the right. Note the smaller size of the female (x 2).

Fig. 2.2 The male moth of *Nudaurelia cytherea cytherea*. Note the large feathery antennae (x 1.5).



CPV, CPV stocks were increased by routinely infecting apparently virus-free larvae reared in the laboratory.

Extraction of virus from *Heliothis armigera*

Three methods were used:

(a) Larvae which died as a result of NPV, GV or mixed NPV, CPV infections were suspended in phosphate buffer pH 7.0 (PB) and permitted to decompose at room temperature. The polyhedral bodies and capsules were released by autolysis and putrefaction.

(b) Alternatively, the dead larvae were homogenized in a mortar, suspended in PB, filtered through cheesecloth and stored at 4°C.

(c) When the larvae contained CPV only, the midguts of the larvae were dissected, ground in a mortar in the presence of PB, filtered through four layers of cheesecloth and stored at 4°C prior to purification.

2.2.2 Source of *Nudaurelia cytherea cytherea*

The larvae were harvested from the pine tree plantations in the Franschoek area, and the pupae were obtained from the Plant Protection Research Institute (PPRI) in Rosebank, Cape. The best source of CPV particles was from the pupae.

The extraction of virus from *Nudaurelia* pupae

Pupae from *N. cytherea cytherea* (Fig. 2.2) were macerated in a Waring blender and the homogenate suspended in PB was filtered through nylon cloth, mesh size 100. The filtrate was clarified by centrifugation at 6500*g* for 10 min. To the supernatant fluid a 6% (*m*/v) polyethylene glycol (PEG) 6000 and 2% (*m*/v) NaCl was added. The mixture was kept at room temperature for 2 h and the precipitate separated by centrifugation at 6500*g* for 10 min in a Spinco 40 rotor. The pellet was re-dissolved in PB, and the solution re-centrifuged as above. The virions in the supernant fluid were sedimented into a pellet by further centrifugation at 50 000*g* for 60 min in the same rotor. Larvae were treated in the same way. Dissected larval midguts were examined separately from the rest of the larval tissues. No organic solvents were used in the extraction procedures, as CPV virions are sensitive to certain organic solvents (Richards and Hayashi, 1971).

2.3 PROPAGATION OF *H. armigera*

Heliothis armigera larvae for the propagation of viruses were reared from pupae originally obtained from the PPRI. The larvae were kept in individual containers to avoid cannibalism and fed on the synthetic diet of Bot (1966) with slight modification by Whitlock (1972). Formalin was omitted from the diet as it has been shown to destroy inclusion bodies (Ignoffo and Garcia, 1968) and Nipagin was retained as a fungal inhibitor.

Composition of Diet:

Wheat germ	24.0 g
Vitamin free casein	4.0 g
Dried Brewer's yeast	22.0 g
Powdered agar	6.0 g
Ascorbic acid	2.5 g
Inositol	0.1 g
Cholesterol	0.1 g
Choline chloride	0.2 g
Nipagin M (Para hydroxybenzoate)	1.2 g
Distilled water	225.0 ml

The medium was prepared by mixing the dry wheat germ, casein and yeast. Nipagin M and cholesterol were dissolved in approximately 20 ml of a mixture of 75% acetone and 25% ether and the solution was stirred into the wheat germ mixture to ensure even mixing of all materials. Organic solvents were evaporated by spreading the mixture out into a thin layer and dry agar was added. Inositol, choline and ascorbic acid were dissolved in 225 ml of water and added. The mixture was heated while being continuously stirred and boiled for 2-3 min to limit microbial growth. The medium was dispensed either in sterile tubes (2 × 10 cm) or in universal containers in 5-10 ml amounts. The containers were held in an upright position to reduce loss of moisture. After cooling, the water which had condensed on the inside of the containers was allowed to evaporate to prevent drowning of the first instar larvae. The tubes were plugged with cotton wool ready for use or capped and stored at -20°C for periods up to six months (Ignoffo, 1965a). Aseptic rearing conditions were maintained by using standard bacteriological techniques.

Eggs on paper pads (Fig. 2.3) were immersed in 0.15% sodium hypochlorite for 5 to 10 min to inactivate surface contaminants (Ignoffo, 1965b). Eggs treated in this way were kept at 24 ± 1°C and about 70% relative humidity. When larvae emerged 3-4 days later they were transferred by means of a fine camel hair brush (sterilized in 0.15% sodium hypochlorite) to containers with the medium. Initially four larvae were placed in one container but after a few days they were separated into individual containers. The incubation temperature was 29 ± 1°C and relative humidity 50%.

Following pupation, the pupae were immersed in 0.15% sodium hypochlorite for 10 to 15 min (Ignoffo, 1965a), placed in glass jars containing fine vermiculite and kept at 24 ± 1°C and about 50% relative humidity. When the adults emerged, 20-25 moths were placed in a specially constructed cage of perspex and nylon-mesh measuring 32 × 26 × 32 cm (Fig. 2.3). Paper pads for oviposition were cut to size and placed between detachable perspex frames to allow for easy removal after oviposition. The adult moths were kept in front of a window to permit natural lighting and fed on 5-10% sucrose solution. The cage was sterilized with sodium hypochlorite solution and washed with distilled water before re-use.

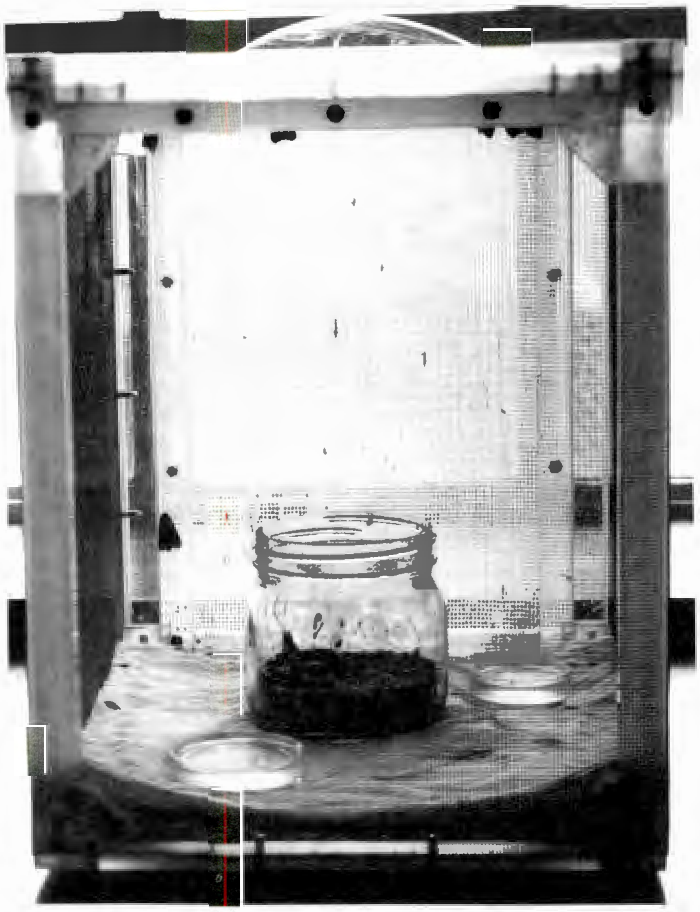


Fig. 2.3 Perspex-nylon-mesh cage used for oviposition of *H. armigera*. Note the detachable paper pads for oviposition, placed facing the light.

CHAPTER 3

PURIFICATION OF INCLUSION BODIES AND VIRUSES

The purification of insect viruses comprises three stages:

(i) The isolation and separation of inclusion bodies from larval debris and bacterial contaminants.

(ii) The partial digestion of inclusion bodies to liberate the virions.

(iii) The purification of the virions.

3.1 PURIFICATION OF POLYHEDRA: METHODS

3.1.1 Centrifugation:

The conditions of centrifugation used throughout this study were as follows:

Inclusion bodies

Polyhedra	......	6 600*g* for 10 min	in a Spinco 40 rotor
GV capsules	......	10 000*g* for 15 min	"

Virus particles

NPV and GV	......	26 000*g* for 30 min	"
CPV	......	26 000*g* for 60 min	"

Details of the rotors and centrifuges used are listed in appendix 2. Both *g* and the rotors used will be mentioned.

3.1.2 Use of Organic Solvents

Polyhedra suspended in distilled water were shaken with an equal volume of Arcton 113 (Imperial Chemical Industries) for several min.

The mixture was centrifuged at 2000 rpm for 30-60 s to separate the water and fluorocarbon phases. The aqueous phase containing the inclusion bodies was removed and the procedure repeated. The aqueous phases were combined and centrifuged to sediment the polyhedra, which were then washed with distilled water.

3.1.3 Centrifugation of Polyhedra through Sucrose Solutions

Polyhedra were routinely purified using methods (i) and (ii) below but for determinations where it was necessary to remove any extraneous protein material, the additional step using method (iii) was included.

Methods

(i) (Khosaka *et al.*, 1971) Partially purified polyhedra suspended in phosphate buffer, pH 7.0 (PB) (1 ml) were layered over (4 ml) 50% sucrose (*m*/v) in the same buffer. The mixture was centrifuged at 9300*g* for 15 min in an SW 50.1 rotor. The pellets of sedimented polyhedra were washed with PB and recentrifuged.

(ii) (Hayashi and Bird, 1970) A suspension of the polyhedral fraction in PB (1 ml) was layered over a discontinuous gradient consisting of 2 ml 51.4% sucrose above 2 ml 75.3% (*m*/v) sucrose in PB. Centrifugation was at 41 400*g* for 45 min in an SW 50.1 rotor. Polyhedra were collected from the interface between the different sucrose solutions and centrifuged at 6600*g* for 10 min in a Spinco 40 rotor, followed by washing in distilled water.

(iii) (Martignoni, 1967) Gradient columns were prepared by floating layers of sucrose solutions at different concentrations in centrifuge tubes in the following order: 80, 78, 76, 74, 72, 70 and 68 (*m*/v) sucrose in PB. Partially purified polyhedral suspensions in PB were applied to the top of the gradient. After centrifugation at 41 400*g* for $2\frac{1}{2}$ h in an SW 50.1 rotor, the polyhedral fractions were collected from their isopycnic level and the refractive indices of the fractions were determined. The polyhedra were sedimented and resuspended in PB.

3.1.4 Results

Polyhedral purification

Larval or midgut extractions were filtered through cheese-cloth to remove large tissue components. Differential centrifugation combined with organic solvent treatment resulted in the removal of larval debris and most bacteria. There was some loss of polyhedra trapped at the water-fluorocarbon interface despite repeated treatments.

Centrifugation of the polyhedral sample through 50% sucrose resulted in the removal of bacteria. Bacteria and debris were located at the buffer-sucrose interface whereas yeast particles were sedimented together with the polyhedra. A relatively pure polyhedral sample was obtained after centrifuging the polyhedral sample treated as in Method (ii). The polyhedra were located at the interface of the sucrose solutions.

During the course of the study it was found necessary to introduce a further purification step in order to remove extraneous protein. The polyhedral suspension was frozen in a dry ice (solid CO_2) - acetone mixture and thawed, before being centrifuged in a 51.4%, 75.3% sucrose gradient. Yeast and viable bacterial counts were reduced. The degree of bacterial contamination being determined by dilutions on agar plates using the drop plate procedure. Plates were incubated at 37 ^{0}C and examined daily. Yeast counts were made using a haemocytometer. A final step of centrifuging the almost pure polyhedral suspension in gradients of 68-80% sucrose reduced any remaining yeast or bacterial counts to relatively low numbers.

In experiments in which CPV polyhedra were counted, difficulty was encountered in separating the polyhedra when the duration of infection was extended beyond six days because polyhedra became tightly packed in

Fig. 3.1 Clumps or bundles of CPV polyhedra before treatment with sodium deoxycholate. Phase microscopy (x 2000).

Fig. 3.2 CPV polyhedra dispersed by 1% sodium deoxycholate treatment for 15 min. Phase microscopy (x 5000).

clusters or bunches (Fig. 3.1). It was extremely difficult to separate the polyhedra and this made accurate enumeration impossible. Attempts to break up these clusters were made as follows:

(i) sonication of 15-s bursts for 1 min;

(ii) shaking of polyhedral suspension in 0.2% sodium deoxycholate (*m*/v) in PB;

(iii) combination of sonication and shaking in 5.0% EDTA (*m*/v), pH 6.0;

(iv) shaking in Triton X;

(v) incubation at 37 ^{0}C in the presence of midgut juices for 2-6 h;

(vi) shaking for 15 min in 1% sodium deoxycholate in distilled water.

Procedures (i) - (v) proved only partially successful in breaking up the clusters, but complete separation of the polyhedral bodies was achieved with procedure (vi) (Fig. 3.2).

3.2 LIBERATION OF VIRIONS FROM INCLUSION BODIES

3.2.1 Alkali digestion

This method is commonly used to liberate virions from inclusion bodies; the concentration of alkaline solutions is chosen to dissolve the polyhedra and capsules, without adversely affecting the liberated viral particles.

Purified polyhedra were dissolved in an alkaline solution ($0.02\ M\ Na_2CO_3$ and $0.05\ M\ NaCl$ 1:1 in CO_2-free distilled water, boiled before use). The pH was adjusted to 10.4-10.6 by adding 1 M HCl. Several periods of digestion, varying with the type of viral inclusion body, were used, once the most suitable conditions for each type had been ascertained. These conditions were:

(i) mixtures of NPV, CPV were digested for 20 min followed by a 30-min period;

(ii) NPV was digested for two 45-min periods;

(iii) CPV was digested for 15-20 min followed by a further period of 20-30 min.

After each digestion step, the sample was diluted in distilled water;

(iv) large quantities of purified CPV polyhedra were digested by dialysing a suspension of the polyhedra against dilute carbonate solution, 0.02 M Na_2CO_3, pH 10.7, for 48 h followed by dialysis against distilled water for 48 h.

Undigested polyhedra were removed by centrifugation. The supernatant fluids were combined for each virus and diluted with PB, and the liberated virions were collected by centrifugation.

3.2.2 Enzyme Digestion

The effects of alkali and trypsin on the digestion of CPV polyhedra were examined.

Purified CPV polyhedra were treated with trypsin (Miles Seravac Co.) 0.05% (*m*/v) in PB, pH 8.0 (pH adjusted with 1 M NaOH). Digestion periods of 20, 40, and 50 min at room temperature were tested. The reaction was stopped by the addition of trypsin inhibitor, 1% (*m*/v) in PB, at different times. Digestion periods of 15 and 30 min, using 0.02 M Na_2CO_3 and 0.05 M NaCl were used concurrently with enzyme digestion on similar quantities of polyhedra. At the termination of digestion, undigested polyhedra and virus particles were centrifuged as described in Section 3.1.1. Samples were examined with the electron microscope.

3.2.3 Results

Alkali digestion

Preliminary experiments using varying times of digestion with

Na_2CO_3, NaCl buffer, pH 10.6 ± 2, were undertaken to determine the optimum conditions for the liberation of virus particles from polyhedra.

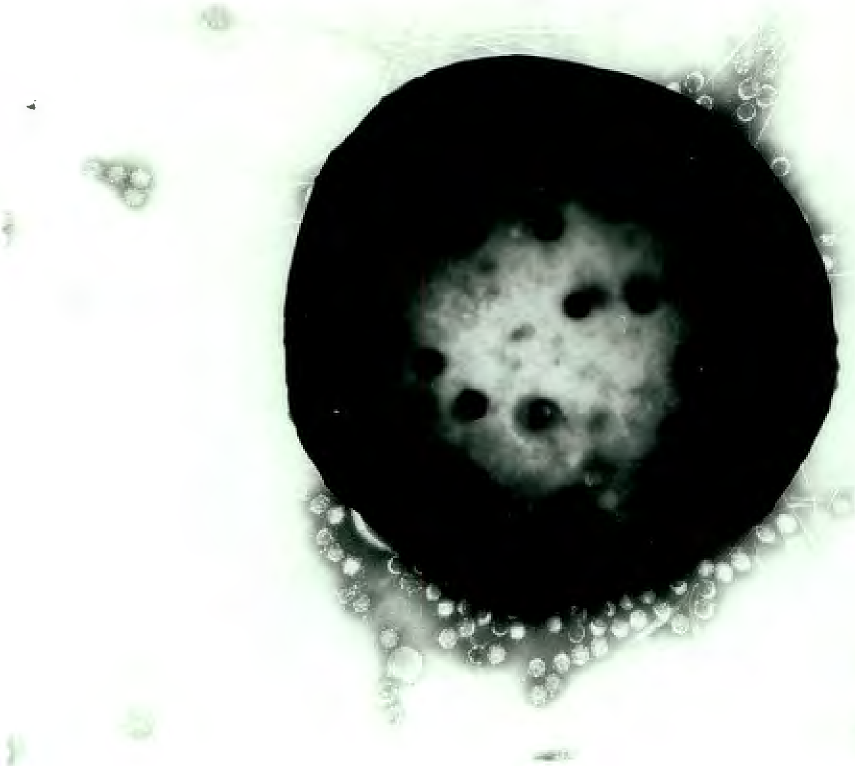
Complete CPV particles were obtained with several short periods of digestion of 15-20 min each. When longer periods were used, liberated particles were damaged. Digestion periods of less than 15 min left large quantities of polyhedral protein undigested. Approximately 80% of CPV polyhedra were digested by dialyzing against 0.02 M Na_2CO_3 solution. The use of this mild solution avoided enzyme damage to the CPV particles.

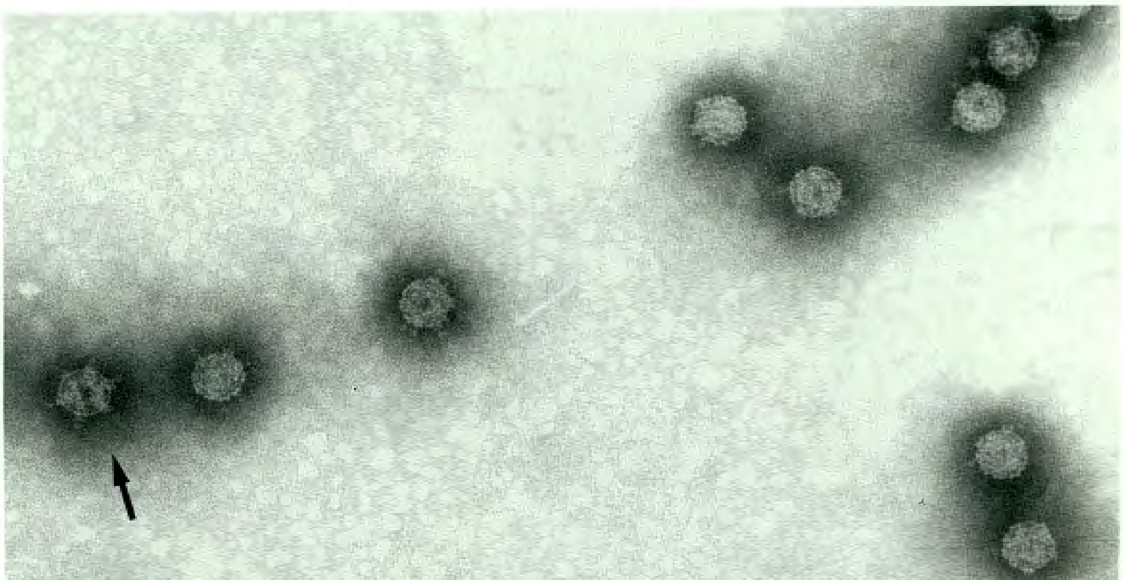
A longer time (45-60 min) was required to digest NPV polyhedra using the Na_2CO_3, NaCl alkaline buffer. This method yielded mainly complete NPV rods. Concentrations of 0.05 M Na_2CO_3, 0.05 M NaCl solution for 1-2 h dissolved the GV capsules of *H. armigera*.

Enzyme digestion

Difficulty was encountered using an alkaline solution to digest the capsule inclusion bodies of GV of *Dionycopus amasus* (Tiger moth) in order to compare its serological properties with that of GV of *H. armigera*. An alternative method was therefore required to digest its inclusion bodies.

Preliminary observations had shown that a proteolytic enzyme isolated from the midguts of control or uninfected *H. armigera* larvae using column chromatography, was similar both in molecular weight and substrate specificity to commercial trypsin. This similarity was ascertained by the Miles Seravac Company using a trypsin assay based on that of Schwert and Takenaka (1955). In this assay, N-benzoyl-l arginine ethyl ester is hydrolyzed at the ester linkage, causing an increase in extinction at 253 nm of 0.003 per min at 25°C, pH 7.6. A comparative study was therefore undertaken to determine whether alkali





or trypsin was more effective in digesting CPV polyhedra.

The effects of short periods of trypsin (0.05%) digestion on polyhedra are shown in Fig. 3.3. After 20 min a number of virions are seen released from polyhedra while others are still partially embedded in the polyhedral matrix. Longer periods of exposure to trypsin, i.e. for 40 and 50 min, resulted in the breakdown of the polyhedral protein with the liberation of undamaged viral particles (Fig. 3.4). The surface structure and arrangement of the projections are clearly visible.

In contrast, similar periods of alkali digestion produced particles that were over-digested. Short periods (15 min) of alkali digestion liberated many CPV particles from digested polyhedra, whereas after 30 min digestion, some damaged particles were seen.

3.3 PURIFICATION OF VIRUS PARTICLES

A combination of differential centrifugation followed by density-gradient centrifugation and zone electrophoresis was used to purify the viruses. Free particles from gut cells and haemolymph, and those obtained from digested polyhedra, were purified in the same way. These techniques were applied to CPV and NPV; studies on GV were undertaken on encapsulated virus only.

3.3.1 Differential centrifugation

A preparative method frequently used to purify free or liberated virus particles is differential centrifugation which utilizes alternate cycles of low and high speed centrifugation.

3.3.2 Rate-zonal centrifugation

Zonal centrifugation utilizes a density gradient in order to prevent convection and to keep particles localized in discrete zones. The rate at which particles sediment while under the influence of a centrifugal force will depend on their size, shape and density in the liquid column. Sucrose is commonly used to make a density gradient. It is readily available in very pure form, is cheap, highly soluble in water, neutral, absorbs little ultraviolet light, and has little effect on viruses apart from osmotic pressure effects.

Method

A 50% sucrose (*m*/v) stock solution was prepared in PB. The stock solution was diluted to obtain a series of dilutions each differing by 5% and ranging from 5-50% sucrose. The gradient was formed by pipetting aliquots of the sucrose solutions of decreasing densities into centrifuge tubes. The densest solution was placed at the bottom of the tube and the more dilute solutions layered sequentially above it. The gradient was left at 4^{0}C overnight for diffusion to take place to ensure approximate linearity of the gradient. Immediately before centrifugation, the virus sample, semi-purified by differential centrifugation, was applied to the top of the gradient. The various gradients and the conditions of centrifugation are listed in Table 3.1. Conditions were chosen so that the relevant zone was situated half-way down the tube at the termination of centrifugation.

TABLE 3.1

SUCROSE DENSITY-GRADIENT CENTRIFUGATION (5-50% SUCROSE) (m/v)

Beckman Rotor Model	Conditions of centrifugation			
	NPV		CPV	
	rpm	min	rpm	min
SW 39	10 000	60		
SW 50.1	15 000	45	22 000	60-90
SW 25.1			22 000	120

Following centrifugation, the gradients were fractionated using an ISCO density-gradient fractionator, Model 640. The gradient was monitored at 260 nm and the ultraviolet absorbing regions were recorded. The fractions containing material from the major peak were diluted with PB and centrifuged at 26 000*g* for 90 min in a Spinco 40 rotor. The pellet was resuspended and washed in distilled water, and after further centrifugation the material was examined in the electron microscope.

3.3.3 Density-gradient zone electrophoresis

Electrophoresis may be defined as the migration of charged particles in an electric field. The surface charge of the particle, whether positive or negative, determines the direction of movement towards either the anode or the cathode. The velocity of the movement depends on the net charge, the voltage gradient of the applied electric field, and the viscosity of the medium. In zone electrophoresis the components of a mixture migrate in discrete zones and are separated according to their charge densities.

The relative electrophoretic mobility, R_ϕ, is the ratio of the distance migrated by the virus sample being studied to the distance migrated by the indicator material, phenol red (van Regenmortel, 1968). The ratio R_ϕ is independent of temperature, concentration, potential gradient and column width.

Method

Reagents: Borate buffer, pH 8.6;

Sucrose solution 40% (*m*/v)

prepared by dissolving 160*g* of sucrose in 200 ml double-strength borate buffer. The pH was adjusted to 8.6 with 1 M NaOH before making the final volume up to 400 ml with distilled water. This step was necessary as sucrose and borate ions form a complex that lowers the pH;

Virus sample, partially purified as described.

The apparatus (Polson and Russell, 1967) is illustrated in Fig. 3.5.

A vertical U-tube containing a wide-bore stopcock on one limb was connected to two electrode vessels (A,B). It has two narrow outlet tubes at its base (E,F). The apparatus was filled with borate buffer, and the 40% sucrose solution was introduced at F to a level above G. The stopcock was closed and the sucrose gradient, made in an apparatus similar to that of Svensson and Valmet (1955) (Fig. 3.5), was introduced at E at a controlled rate. The concentration gradient formed is of the logarithmic type; its operative range is approximately linear. This allows a constant migration of the particles under standard conditions.

Excess buffer was removed from A, and reversible Ag/AgCl electrodes

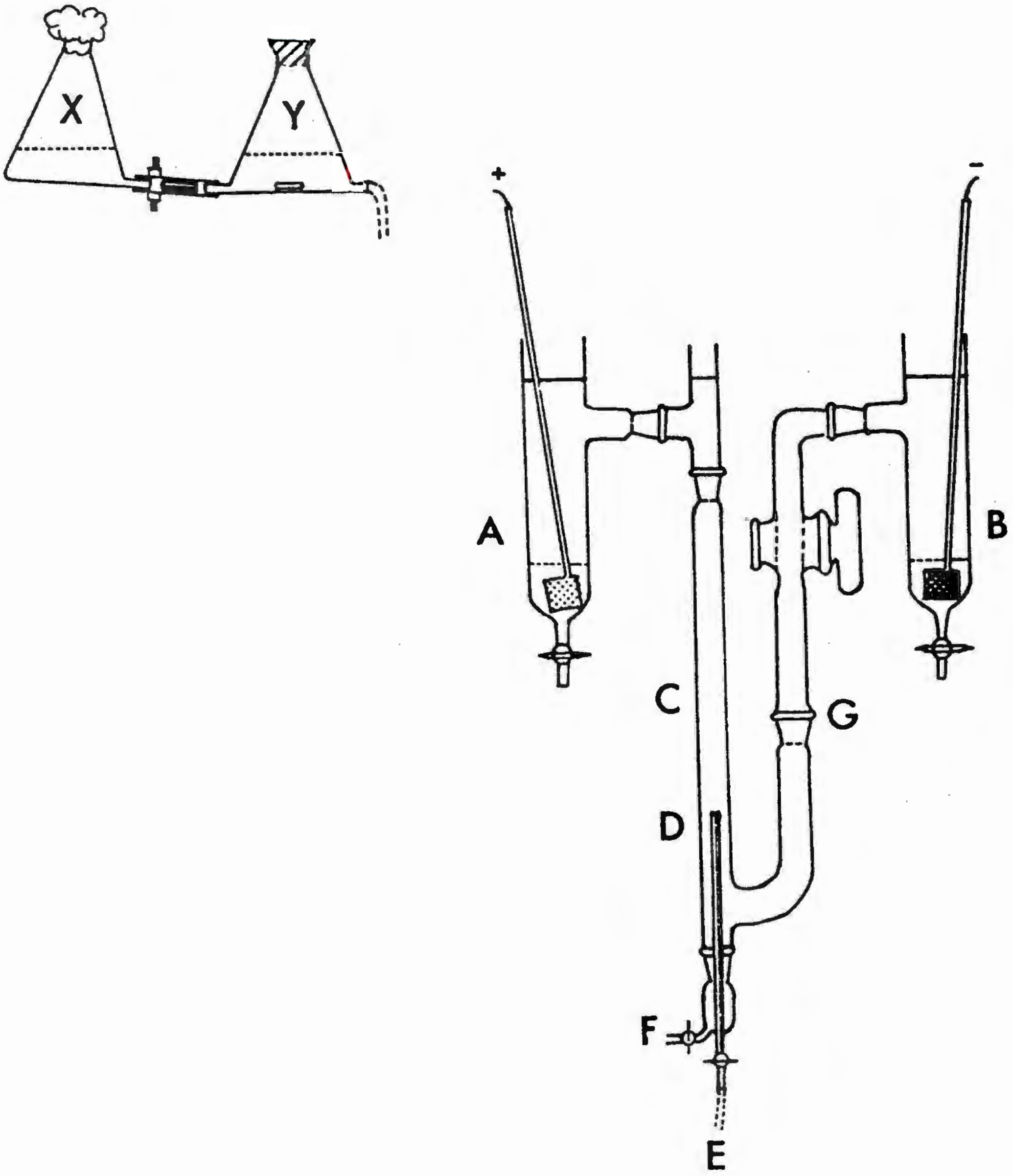


Fig. 3.5 Apparatus for density-gradient zone electrophoresis:

A and B, electrode vessels;
C, electrophoresis column, containing a capillary, D, and with outlet at F;
E, capillary outlet;
G, level of 40% sucrose;
X and Y, gradient-forming flasks;
X contains 160 ml of 40% sucrose, and Y contains 170 ml of buffer and is positioned above a magnetic stirrer. X and Y are connected with a capillary tube.

were positioned in the electrode vessels A and B and covered with saturated NaCl solution. The virus sample, together with 37% sucrose and phenol red as reference substance, was introduced through E with a syringe. The column was balanced by adjusting the buffer volume in A or B, and was equilibrated by air cooling for several hours. The stopcock was opened and approximately 20 mamps at 3.5 *V*/cm was applied to the column. Electrophoresis proceeded for 18-20 h. The stopcock was closed and the gradient was examined in the light of an argon mercury vapour lamp.

To prevent the tailing of virus while fractions were being collected from the column, samples, equivalent to 1 cm column length, were removed both from the top end of the column, and from the capillary at the base. Each sample after dilution with PB was washed free of sucrose by centrifugation at 41 000*g* for 90 min in a Spinco 40 rotor, followed by similar treatment with distilled water and centrifugation at 26 000*g* for 30 min in the same rotor. The pellets from the final centrifugation were examined in the electron microscope to detect the presence of virus.

3.3.4 Negative Staining for Electron Microscopy

Purified virus samples were sedimented by centrifugation, and excess moisture was drained from the pellet. The virus pellet was resuspended in a small volume (approximately 0.1 ml) of distilled water. A drop of suspension was mixed on a glass slide with an equal quantity of 2% (*m*/v) potassium phosphotungstic acid (PTA) (adjusted to pH 6.0 with 1 M KOH). A drop of this mixture was placed on a 300 mesh, formvar carbon-coated grid, and excess sample was withdrawn with filter paper. The grid was placed in a Siemens Elmiskop 1A electron microscope and examined at a suitable magnification and at an accelerating voltage of

80 KV.

3.4 RESULTS

Initial samples of NPV-infected *H. armigera* larvae obtained from the Plant Protection Research Institute (PPRI) were found on examination to contain a mixture of NPV and CPV with low concentrations of CPV. As this was the only material then available, the two viruses had to be separated before any further studies could be undertaken. Since this separation is central to the entire thesis, it will be described first. After the viruses had been separated, they were propagated in laboratory-reared larvae. Details of their purification will be described in Sections 3.4.2 and 3.4.3.

3.4.1 Isolation of CPV From a Mixture of NPV and CPV

Initial attempts to separate the viruses were either unsuccessful or only partially successful and are briefly mentioned below.

Preliminary experiments

Attempts to separate the polyhedra of NPV and CPV using gradient centrifugation, both rate-zonal and isopycnic (Van der Geest, 1968; Martignoni, 1967) were unsuccessful. The following methods were applied to virions liberated from polyhedra by alkali treatment. The degree of separation in each case was estimated by electron microscopy.

(i) Sucrose-gradient centrifugation yielded a zone of NPV particles contaminated with some CPV, but no zone of CPV.

(ii) Isopycnic centrifugation in CsCl (Polson and Levitt, 1963) and in potassium tartrate (McCrea *et al.*, 1961) yielded a similar result.

(iii) Filtration using gradocol filters (made in this laboratory) of pore sizes 219, 251, and 377 nm was unsuccessful.

(iv) Centrifugation in the thin-layer rotor of Polson and Kiefer (1975) resulted in a separation of about 50% CPV from NPV. The CPV fraction was contaminated with empty nucleocapsids of NPV and several whole particles.

(v) Zone electrophoresis resulted in the continued presence of numerous empty NPV nucleocapsids and some NPV particles in the CPV zone.

(vi) Column chromatography using columns of 4B Sepharose (Pharmacia) or 2% agarose pearls (Hjertén, 1964) was unsuccessful; however, after using a column of 2% agarose granules, the CPV zone contained approximately 90% CPV and 10% NPV.

(vii) Impeditive electrophoresis (Polson and Russell, 1967) was performed in which a stepwise agarose gradient electrophoresis was used; the agarose concentrations ranged from 0.3% to 10%. NPV particles were trapped in the 2% agarose layer and CPV in the 5% agarose. Although there was apparently a 5% contamination of NPV inner particles in the CPV zone, separation of approximately 95% of CPV particles was obtained.

Complete separation of CPV and NPV virions was achieved by a combination of techniques as follows:

Differential centrifugation

To ascertain the conditions necessary for optimal separation of the virions by differential centrifugation, the sedimentation coefficients (*s*) of the virions present in a mixture of NPV and CPV were first determined. (See Section 7.2.2): They were 389 and 1188 S for CPV and

NPV respectively. Using these values together with the known minimum time (60 min at 59 000*g* in a Spinco 40 rotor) required to sediment *Burnupena cincta* haemocyanin ($s_{20.w}$ = 100), it was possible to calculate the most effective period of ultracentrifugation required to remove most of the NPV with minimal loss of CPV from the suspension. This period was calculated to be 13 min at 26 000*g*. Three repeated centrifugations at this speed removed the bulk of NPV particles, but further centrifugation damaged CPV particles. The final supernatant fluid was centrifuged at 26 000*g* for 40 min to sediment CPV into a pellet.

Electron micrographs of the virus contained in the initial mixture (Fig. 3.6), in the pellet (Fig. 3.7), and in the supernatant fluid (Fig. 3.8), are shown. The majority of the contaminating complete NPV virions and empty NPV nucleocapsids remaining in the supernatant fluid were then removed by zone electrophoresis.

Zone Electrophoresis

Zone electrophoresis was performed on a CPV fraction, partially purified by differential centrifugation. The column was fractionated as described in Section 3.3.3, and virus recovered from the diluted aliquots by centrifugation at 26 000*g* in a Spinco 40 rotor for 40 min. Since CPV migrated with the phenol red, no zone of light scattering was visible. Its position was determined by electron microscopy. Table 3.2 indicates the relative electrophoretic mobilities of the different virus particles expressed as R_ϕ values. The majority of complete NPV particles were well separated from the faster migrating CPV, but the electrophoretic heterogeneity of the empty NPV mucleocapsids resulted in some contamination of the CPV zone. The remaining contaminating particles were removed by treatment with specific NPV antibody.

Fig. 3.6 The original mixture of NPV (N) and CPV (C). Note the icosahedral CPV particles, and the projections visible on several particles. Rod-like NPV particles are seen in various stages of digestion:

N_1, NPV particle within virus membrane;

N_2, particles with partially digested virus membrane;

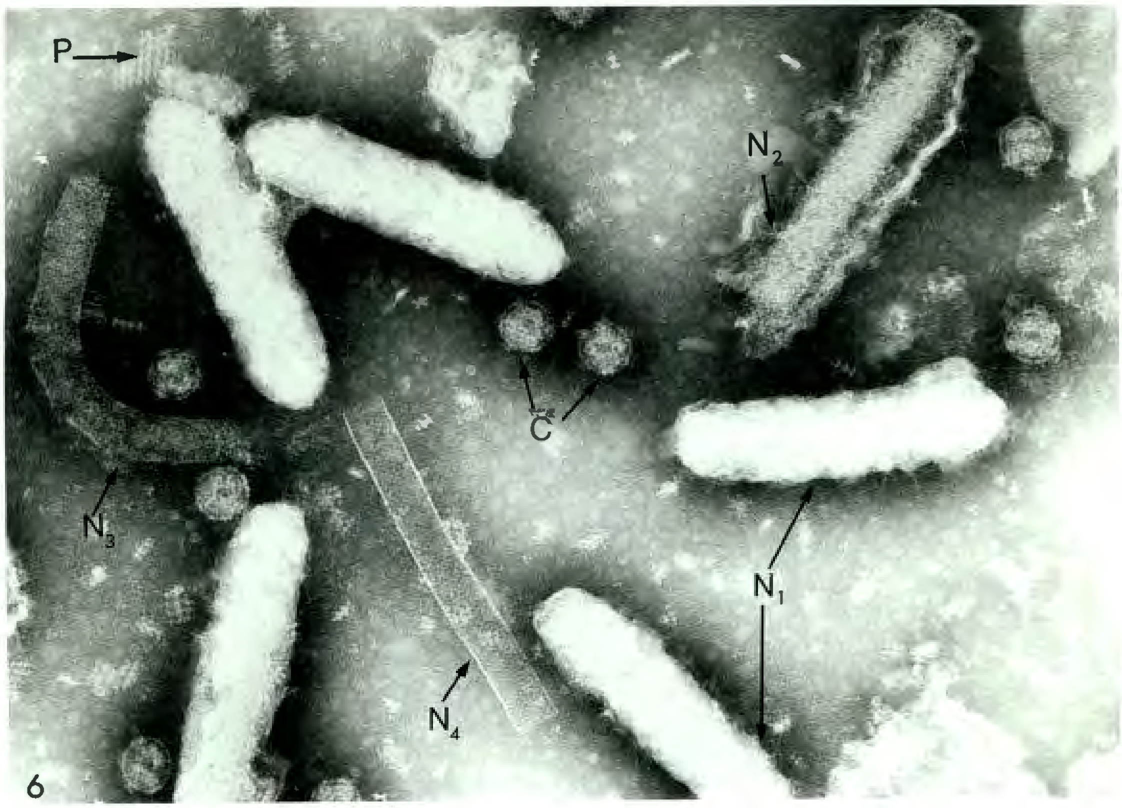
N_3, NPV capsid, and

N_4, empty capsid or nucleocapsid penetrated by the electron-dense stain.

Note the polyhedral protein (P) in lattice-like aggregates (x 80 000).

Fig. 3.7 Partial separation of NPV and CPV after three consecutive centrifugations at 26 000*g* for 13 min in an SW 50.1 rotor. The resuspended pellet consisted predominantly of NPV particles (x 80 000).

Fig. 3.8 The supernatant fluid after three ultracentrifugations at 26 000*g* for 13 min to remove NPV particles. Note the predominance of CPV (x 80 000).


P
N2
C
N3
N1
N4
6

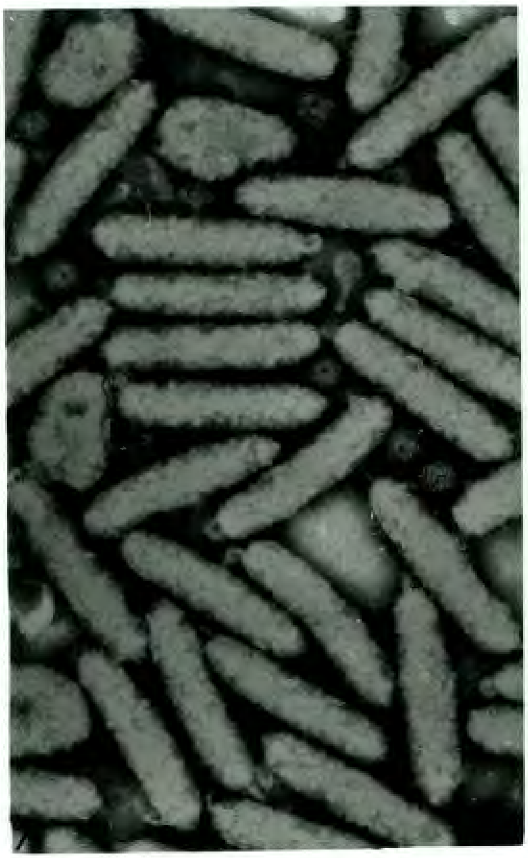

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TABLE 3.2

ELECTROPHORETIC MOBILITY OF THE DIFFERENT VIRUSES RELATIVE TO DISTANCE MIGRATED BY PHENOL RED

Virus	Mean R_ϕ	Number of runs
H. armigera		
* CPV_{A1}	1.0	3
** CPV_{A2}	1.0	8
NPV virus particles	0.59	2
NPV empty capsids	0.82	3
N. cytherea cytherea		
CPV	0.56	4
ε particle	0.64	2

* CPV_{A1} - field isolate

** CPV_{A2} - laboratory-passaged isolate

Treatment with specific NPV antibody

The virus sample was suspended in phosphate buffered saline (PBS) and treated with a 1:10 dilution of NPV antiserum. After thorough mixing, the sample was kept overnight at 4°C. The sample was diluted with PBS and centrifuged at 17 600*g* for 15 min in an SW 50.1 rotor to remove NPV complexed with antibody. The supernatant was centrifuged at

Fig. 3.9 A sample of partially separated CPV and NPV after treatment with antibody to NPV. Note the clumped NPV particles in contrast to the dispersed CPV particles (x 80 000).

Fig. 3.10 CPV particles isolated from the midguts of *H. armigera* larvae that had been infected with the final CPV sample treated with NPV antiserum. The projections are easily visible with a knob at the tip of some projections (arrowed) (x 120 000).

31 600*g* for 40 min in the same rotor to sediment CPV particles. An electron micrograph of NPV complexed with antibody is shown in Fig. 3.9.

The purity of the final CPV suspensions was assessed by infecting virus-free *H. armigera* larvae with the material obtained after antibody treatment, i.e. with the supernatant containing the remaining CPV virions, after removal of the NPV virions complexed with antibody. Six to eight days after ingestion of infective material, the larvae were killed and the midguts dissected. The midguts and the remainder of the larval tissue were extracted separately and examined for the presence of virus by electron microscopy. Only CPV virions, both free and occluded, were found in extracts of the midgut (Fig. 3.10). No NPV particles were found either in the remainder of the tissues or on further passage of this CPV stock. Only a single sedimenting peak with S equal to 389 was found on examination in the analytical ultracentrifuge, confirming the earlier assumption that the sedimentation coefficient for CPV was 389 S.

The infectivity of antibody-complexed NPV particles was retained, as seen by feeding this suspension to *H. armigera* larvae, which consequently died.

3.4.2 Purification of CPV from *H. armigera*

CPV obtained by passage in laboratory-reared larvae was purified as follows:

Undigested polyhedra, bacterial contaminants and degraded polyhedral protein were removed by differential centrifugation. This procedure was followed by repeated centrifugations in 5-50% (*m*/v) sucrose gradients. Following centrifugation, the gradient was monitored at 260 nm. A typical ultraviolet absorption profile of the material on the gradient is shown in Fig. 3.11.

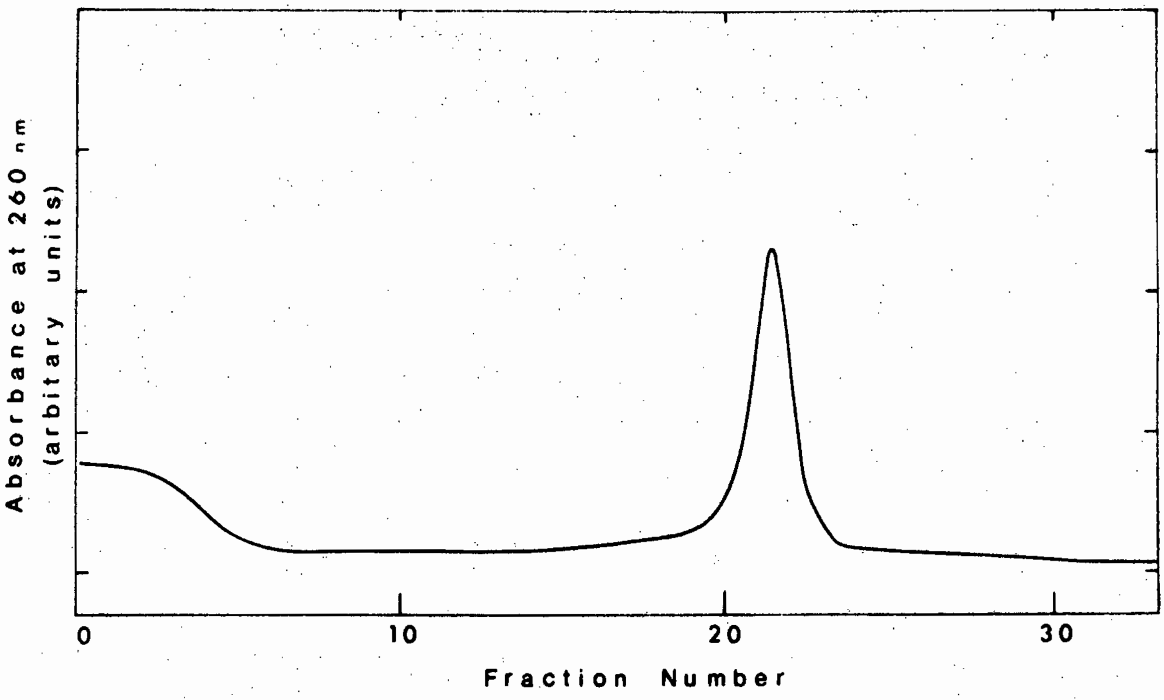


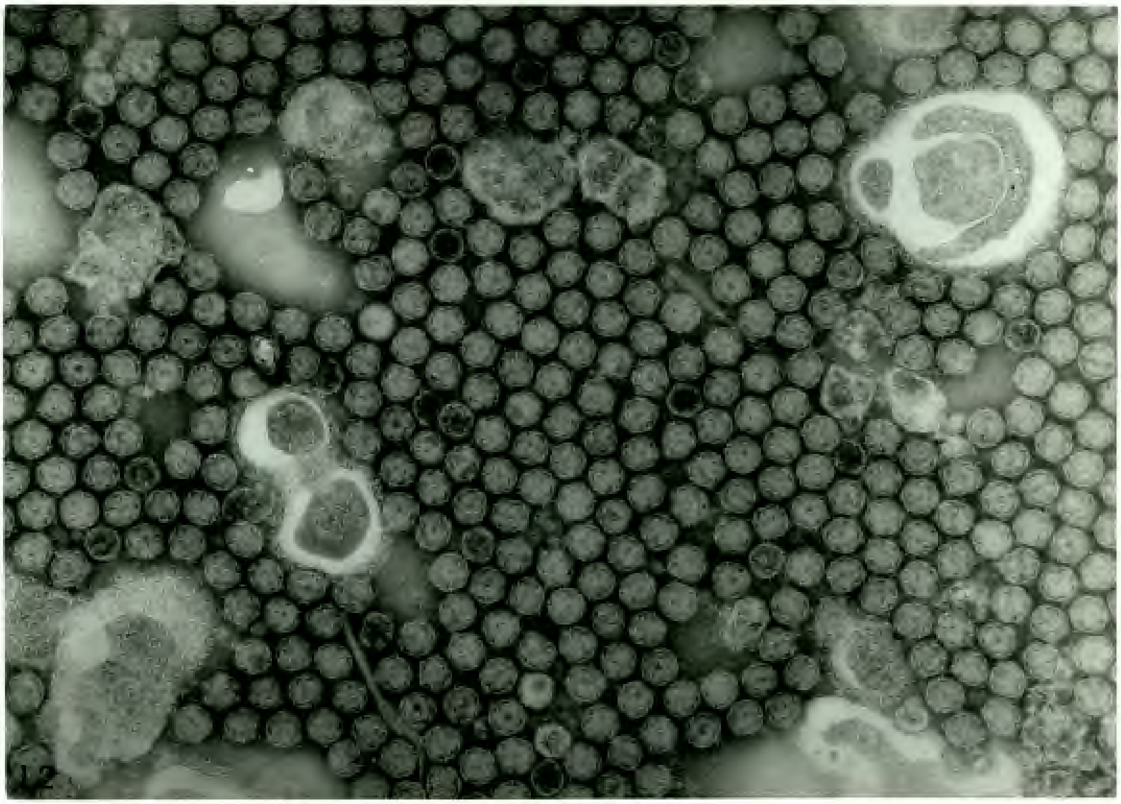
Fig. 3.11 Rate-zonal sedimentation of CPV of *H. armigera* in 5-50% sucrose gradients. Sedimentation is from left to right.

Two ultraviolet absorbing zones are seen. The major peak forms an opalescent band consisting of mainly intact and some empty virus particles as revealed by electron microscopy (Fig. 3.12). The minor peak on the ultraviolet profile, situated near the top of the gradient, contained a few empty virions and dissolved polyhedral protein. After two cycles of sucrose density-gradient centrifugation, only a single homogeneous peak of virus particles was obtained, corresponding to the major peak on the ultraviolet profile. Electron microscopy of CPV particles obtained after centrifugation in sucrose density gradients showed that many of the hollow projections of the virus particles were

Fig. 3.12 *H. armigera* CPV purified by sucrose density gradient centrifugation. Note the absence of projections on many particles (x 80 000).

Fig. 3.13 Purified CPV from *H. armigera* recovered from a fractionated zone electrophoresis column. Note the close packing and absence of projections (x 80 000).

Fig. 3.14 *H. armigera* NPV purified by sucrose density gradient centrifugation. Complete particles or capsids are seen, no empty capsids are present in this fraction (x 80 000).



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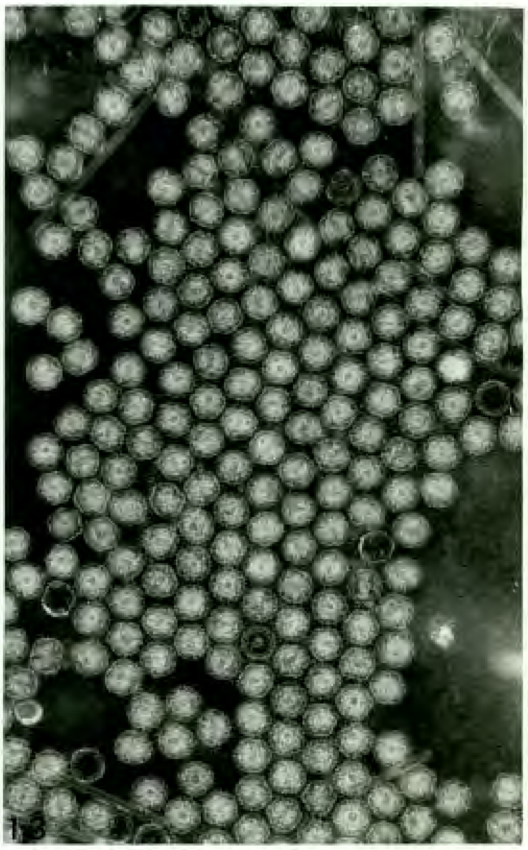

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no longer visible (Fig. 3.12).

Further purification of CPV particles was achieved by zone electrophoresis. CPV particles have a high electrophoretic mobility at pH 8.6 and migrated with the indicator phenol red, with R_ϕ of 1.0 (Table 3.2). They were well separated from debris and other contaminating material. Figure 3.13 shows an electron micrograph of the fraction containing CPV collected after zone electrophoresis.

The particle diameter measured on electron micrographs averaged 59.3 ± 3.2 nm (37 measurements).

3.4.3 Purification of NPV from *H. armigera*

After digestion of NPV polyhedra, virus particles were purified by differential centrifugation followed by centrifugation in sucrose density gradients. Gradients were monitored and fractionated as described for CPV (3.4.3). Electron microscopy revealed the presence of purified virions recovered from the band of opalescence (Fig. 3.14).

Further purification of NPV was achieved by zone electrophoresis. Two light scattering zones were observed in the column after electrophoresis, one zone with R_ϕ value of 0.59, and the other with R_ϕ of 0.82 (Table 3.2). Electron microscopy showed that one zone, ($R_\phi = 0.59$), consisted mainly of complete NPV particles, whereas the other, ($R_\phi = 0.82$), contained empty nucleocapsids.

Dimensions of virus particles were found to be 368 ± 13 x 90.5 ± 4 nm (15 measurements) on electron micrographs.

3.4.4 Purification of CPV from *Nudaurelia cytherea cytherea*

Viral material of *N. cytherea cytherea* was extracted as described

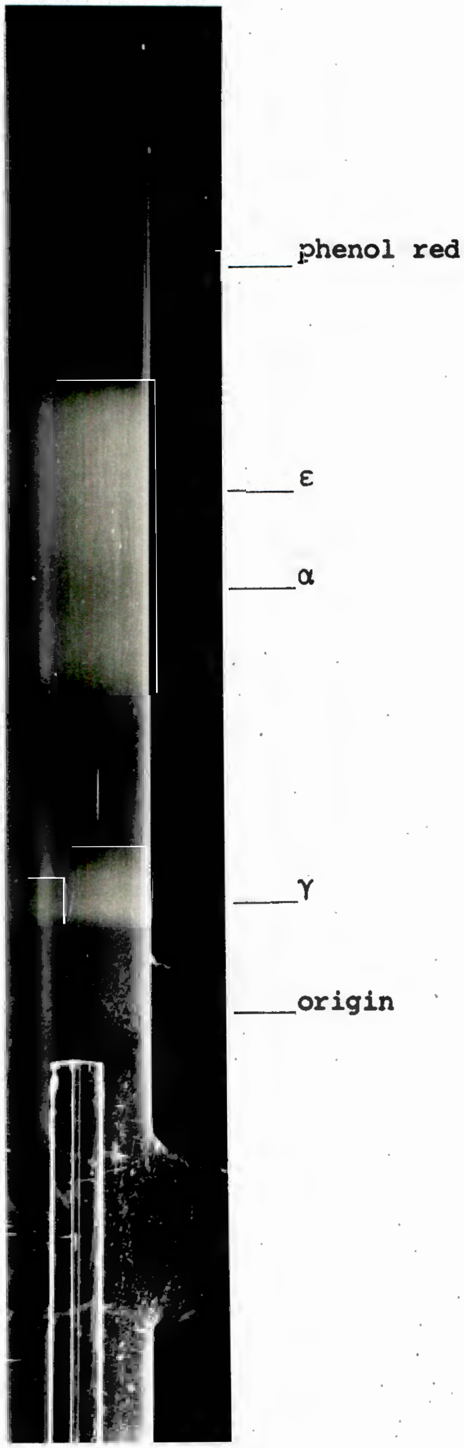
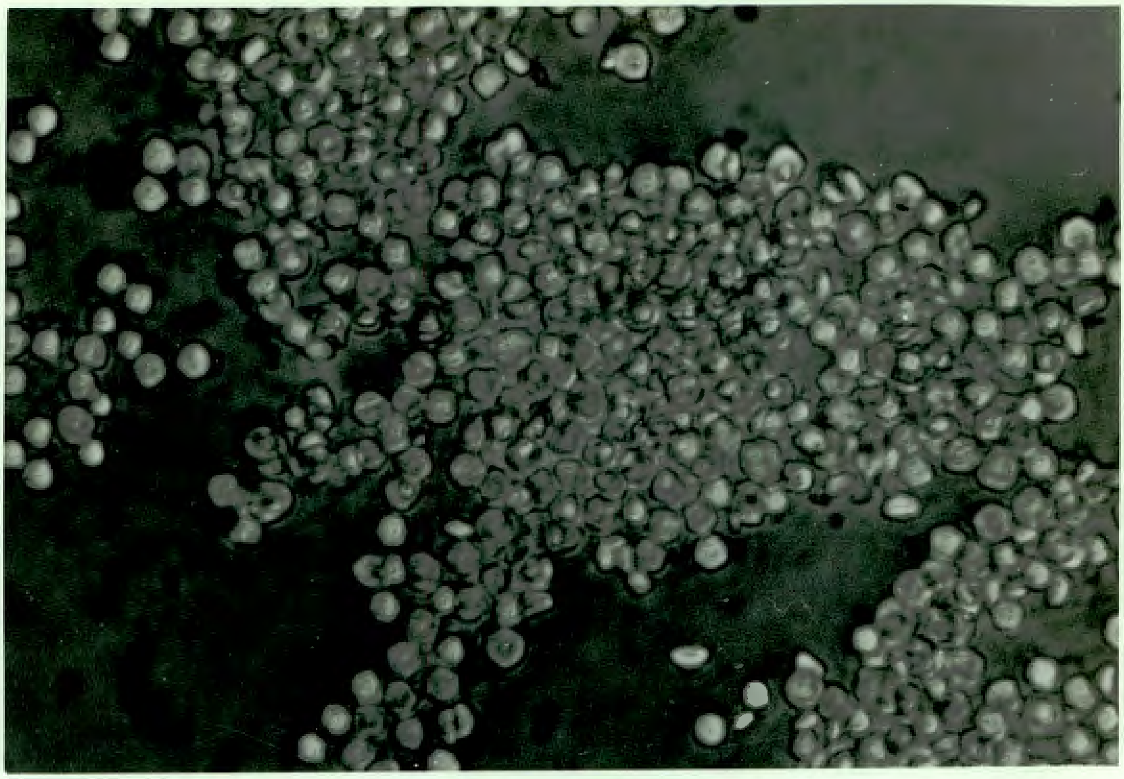


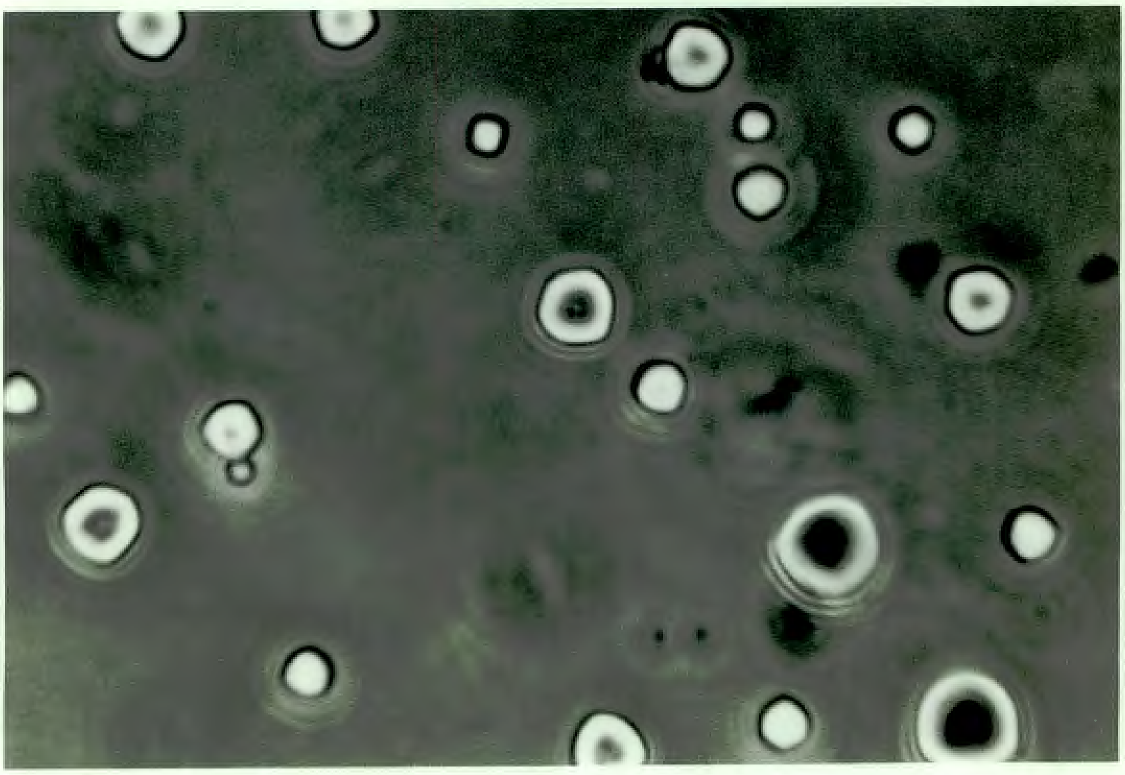
Fig. 3.15 Zone electrophoresis column after electrophoresis of *N. cytherea cytherea* extracts. The positions of the various viruses corresponding to the different opalescent zones are indicated. The phenol red appears as a dark band in the photograph.

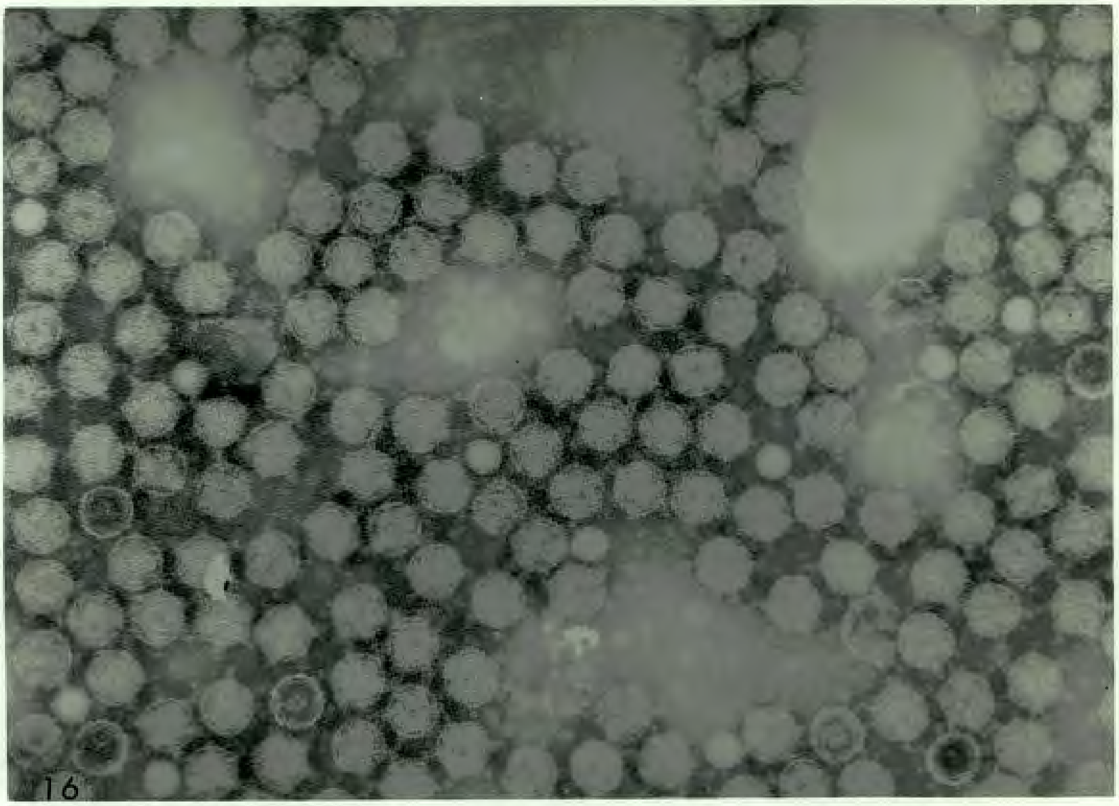
Fig. 3.16 Electron micrograph of the *N. cytherea cytherea* CPV (α) particles recovered from the zone electrophoresis column. Note the presence of smaller ε particles (x 120 000).

Fig. 3.17 Electron micrograph of *N. cytherea cytherea* ε particles recovered from the upper part of the CPV zone. Note the presence of some CPV (α) particles (x 120 000).

Fig. 3.18 Purified CPV of *N. cytherea cytherea* after isopycnic centrifugation in CsCl gradients. Note the projections on the CPV particles. A single ε particle is still present (x 120 000).



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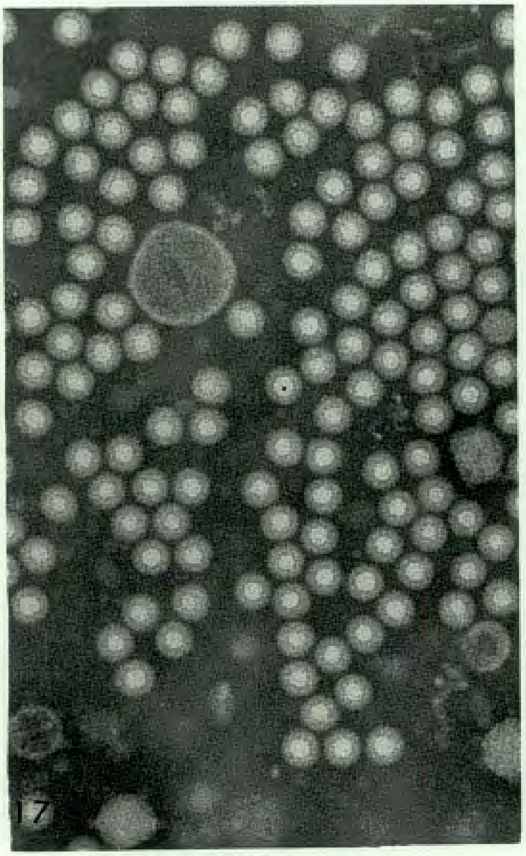
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in Section 2.2.2. In order to isolate the CPV or α particle from the other viruses, the material was subjected to zone electrophoresis. A photograph of the light scattering in the column after electrophoresis is shown in Fig. 3.15. The CPV and ε particles of *N. cytherea cytherea* were well separated from the β, γ and δ viruses, but the α and ε particles were not completely separated from each other (Figs. 3.16 and 3.17). After further treatment by isopycnic centrifugation in CsCl gradients (see Section 7.1.2), the CPV particles were successfully purified (Fig. 3.18).

A particle diameter of 63.7 ± 2.8 nm (10 measurements) was found on electron micrographs.

3.5 DISCUSSION

Purified preparations are essential for detailed characterisation of virus particles and for analysis by serological techniques. In contrast, less purified or even unpurified suspensions may be suitable in field work (Ignoffo, 1964), or for certain types of bioassay (Magnoler, 1968). In this study, purification procedures for polyhedra and virions varied according to the purpose of the experiment.

Differential centrifugation combined with the use of organic solvents yielded polyhedra and capsules sufficiently pure to infect larvae. For protein analysis and antiserum production, extraneous protein was removed by centrifugation in sucrose-density gradients, and in some instances also by isopycnic centrifugation in a sucrose gradient. The effectiveness of the purification was monitored by microscopic examination and by bacterial counts. The bacterial counts were reduced to a low level similar to those reported by Padhi *et al.* (1974).

As part of the technique for the purification of virions it was necessary to release the viral particles from the polyhedra. Alkali treatment of the inclusion bodies has been well documented, e.g. Bergold (1947); Smith and Wyckoff (1951). Since conditions for the alkali digestion were critical, particular attention was paid to alkali concentration, the time of digestion and the repetition of the exposures. Hukahara and Hashimoto (1966) used carbonate buffer for CPV of *B. mori* and found pH and volume to be important considerations. However, this method was not satisfactory for liberating viruses from the polyhedra of certain families such as Nymphalidae, Arctiidae, Hepialidae, Lasciocampidae and Nototidae (Cunningham and Longworth, 1968). Hills and Smith (1959) found that short liberation periods were more satisfactory for recovering intact virus particles, whereas Smith (1967) noted that CPV polyhedra dissolved less readily than those of NPV.

In the present study a modification of Bergold's method (1963b) was successful. Short digestion periods were effective for CPV polyhedra, but NPV polyhedra required longer periods. For most of the experiments in this study, alkali treatment was satisfactory for the liberation of undamaged CPV particles. Enzyme digestion was more easily controllable, but the continued presence of enzyme inhibitor prohibited its general use.

Previous studies have shown that NPV polyhedra are unaffected by enzymes. However, Gipson and Scott (1975) found that in thin sections, pepsin, subtilisin and pronase digested the polyhedral protein but not the polyhedral membrane. Trypsin, on the other hand, had no effect on its own, but when combined with DNase removed the polyhedral protein and virion nucleocapsids. This finding was similar to that of Thomas and Williams (1961), who used a mixture of DNase and trypsin to digest the nucleoprotein of tipula iridescent virus. Bergold (1958) cited the work of Zalmanson, which showed that polyhedra are resistant to papain, trypsin and pepsin at pH 3.3 to 4.0. This is not unexpected since the optimum pH for trypsin is 8.0, which was the pH used in the present study.

The effect of enzymes may be related to the presence or absence of a polyhedral membrane, a subject which has been extensively debated in the literature (Bergold, 1958; Harrap, 1972a). Convincing evidence of the existence of a polyhedral membrane for NPV polyhedra was provided by Harrap (1972a). Thin sections and electron micrographs of mature CPV polyhedra revealed what appeared to be a membrane surrounding these polyhedra. It is not clear, therefore, why CPV polyhedra were digested by trypsin in the present study.

Separation of CPV and NPV

Multiple infections with CPV and NPV have been described in Lepidoptera by Vail *et al.* (1972) and Smith and Xeros (1953b). This phenomenon was also encountered in *H. armigera* during the course of the present study.

Attempts to separate the polyhedra of these viruses by sucrose density-gradient centrifugation proved unsuccessful, possibly because the polyhedra varied in size and density. In addition, there were large numbers of NPV polyhedra relative to CPV polyhedra. Later studies also revealed that NPV and CPV polyhedra are similar in size, 3 to 7 days after infection; the time when NPV infection usually kills the larvae.

Separation of the virions was not achieved in the preliminary experiments because of the low concentrations of CPV present, and the nature of the particles themselves. Mutual aggregation of the two viruses and the subsequent trailing of NPV resulted in inadequate separation of the mixture of viruses in methods such as column chromatography and zone electrophoresis. CPV was, however, successfully isolated and purified from a mixture of released CPV and NPV virions by a combination of differential centrifugation, zone electrophoresis and complexing with antibody.

Differential centrifugation was the most convenient preliminary isolation method, and yielded CPV in the most intact state. Despite the difference in sedimentation coefficients, the two viruses could not be completely separated by this method. This may be attributed to the effect of sedimentation on the walls of the cell due to the cylindrical shape of the tube, with convection currents playing a part; and also variation in the physical properties of the particles such as the presence of empty nucleocapsids resulting in association of particles. Increasing the number of centrifugation cycles increased the number of damaged CPV particles.

When differential centrifugation was followed by zone electrophoresis, a relatively pure preparation of CPV was obtained. Zone electrophoresis in a density gradient has been used for the classification of enteroviruses on the basis of their electrophoretic mobilities (Polson and Deeks, 1962) and for the purification of plant viruses (Van Regenmortel, 1964). It was also highly efficient in this work for separating CPV from the small numbers of NPV. Enveloped NPV and CPV virions differ sufficiently in their electrophoretic mobilities to allow good separation in a zone electrophoresis column.

Adherence of any polyhedral protein on the surface of virions would affect the surface charge of the particles and could result in contamination of one zone with another. Some empty NPV nucleocapsids did migrate at a similar rate to that of CPV; this may be due to the loss of surface protein and exposure of differently charged groups. In addition, the influence of the nucleoprotein on the charge of the whole viral particle will be removed once the nucleoprotein has been released, as in empty nucleocapsids. A change in tertiary structure could occur with reorientation of charged groups to the water phase. This may result in a higher negative charge which would increase the movement of empty capsids towards the positive pole.

Zone electrophoresis, in conjunction with differential centrifugation, yields CPV sufficiently pure for most studies. However, for infectivity studies and the production of antiserum, a pure sample was required, necessitating the additional adsorption treatment with anti-NPV antiserum. After removal of the NPV-antibody complexed material, passage in larvae of the remainder of the material, resulted in an infection from which only pure CPV was harvested.

Purification of Virions

Following the separation of the two viruses, larger quantities of viral material became available after passage in laboratory-reared larvae. These viruses were purified using standard techniques.

Density gradient centrifugation has been used in the purification of CPV (Hayashi and Bird, 1970; Payne, 1971) but only rarely in the case of baculoviruses (Harrap, 1972b; Harrap and Longworth, 1974). Previously, serological comparisons on NPV had been undertaken on preparations purified by differential centrifugation alone.

A combination of differential and rate-zonal centrifugation was

CHAPTER 4

SEROLOGICAL RELATIONSHIPS BETWEEN VIRUSES

Although insect viruses are good antigens, few attempts have been made to identify them serologically. In this study, the serological relationships between various viruses were investigated by means of the techniques of immune electrophoresis and immune electron microscopy. Immunodiffusion reactions were used in the measurement of diffusion coefficients (Section 7.4).

4.1 METHODS

4.1.1 Preparation of Antisera

Control sera were obtained from rabbits prior to immunization. Animals received two intramuscular injections, a week apart, of a suspension of purified virus mixed with Freund's complete adjuvant. Two weeks after the second injection, they received a final injection containing virus only. Animals were bled from the ear at weekly intervals. After the blood had been allowed to clot and left standing for several hours at room temperature or overnight at 4°C, the serum was decanted and clarified by centrifugation at 2000 rpm for 30 min. Aliquots of sera were stored at -20°C without preservative or at 4°C with added merthiolate (0.001% final concentration).

Antisera to the following viruses were employed in this study:

(i) CPV_{A1} from *H. armigera* (field isolate);

(ii) CPV_{A2} from *H. armigera* (isolated from laboratory-reared larvae, Section 8.2.3);

(iii) NPV from *H. armigera*;

(iv) GV from *H. armigera*;

(v) CPV from *Malacosoma disstria*;

(vi) CPV from *Bombyx mori*:
  (a) strain with icosahedral polyhedra;
  (b) strain with hexahedral polyhedra.

Antisera to viruses (i) and (ii) were prepared in this laboratory. Antisera to (iii) and (iv) were provided by D. Tripconey, and antisera (v) and (vi) were gifts from Dr. W.C. Richards (Canada) and Dr. S. Kawase (Japan), respectively.

4.1.2 Immune Electron Microscopy

This technique was used to study the serological relationships among a variety of viruses.

For each virus-antibody system, suitable proportions of the reactants were established by serial dilutions of the antiserum in phosphate buffer ranging from undiluted to 1/50. Equal volumes of virus suspension and diluted antiserum were mixed, incubated at 37°C (for 30-60 min) and then kept overnight at 4°C. Phosphate-buffered saline (PBS) was added to the samples, which were centrifuged at 9300*g* for 15-20 min in the SW 50 rotor. The pellet was washed with distilled water to remove excess protein and after recentrifugation it was examined by electron microscopy. The supernatant fluid was centrifuged at 37 000*g* for 15 min and 45 min for NPV and CPV respectively, in the same rotor and the samples were examined by electron microscopy. Control samples were treated in the same manner using pre-immune serum.

4.1.3 Immune Osmophoresis

Immune osmophoresis is performed by placing the antibody on the anode side and the antigen on the cathode side of an agar-coated slide. The reactants are placed 5-10 mm apart, on the same electrophoretic axis. The antigens move towards the anode at pH 8.6, while the antibody moves in the opposite direction because it is more subject to electroendosmosis. The net result is that the reactants migrate towards each other more rapidly than by diffusion and that lines of precipitation appear in a very short time.

Reagents: Buffer, Barbital-glycine-tris, pH 8.6
Agar Ionagar No. 2, Oxoid Ltd., London
Staining solution - filtered before use:

Coomassie blue	5 g
Glacial acetic acid	100 ml
Ethanol 96%	450 ml
Distilled water	450 ml

Destaining solution:

Ethanol 96%	450 ml
Glacial acetic acid	100 ml
Distilled water	450 ml

Washing solutions:

Normal saline
Distilled water

Method:

Glass plates (6 × 10 cm) were uniformly coated with 10 ml of 0.5% (*m*/v) of Ionagar No. 2 in glycine-tris buffer, resulting in a gel thickness of approximately 1.5 mm. When the agar had cooled, two parallel lines of 2 mm circular holes were punched 5 mm apart in the gel with a pasteur pipette. Any fluid was removed before application of the sample. The gels were placed in an electrophoresis tank and equilibrated with a current of 1-2 mamp for 5 min. Samples were inserted into the previously prepared holes with a finely drawn out pasteur pipette, antigens in the row nearest to the cathode and antiserum closer to the anode. A potential of 2 V/cm was applied and electrophoresis continued for about 15 h. After the current was stopped, the gels were kept in a moist chamber for 3 h to allow further development of the precipitin lines. The gels were pressed and washed in saline (3 × 15 min), washed once in distilled water for 15 min, and dried in hot air. The immunoprecipitates were stained in Coomassie blue solution for 10 min and cleared in destaining solution.

4.2 RESULTS OF SEROLOGICAL STUDIES

4.2.1 Immune Electron Microscopy

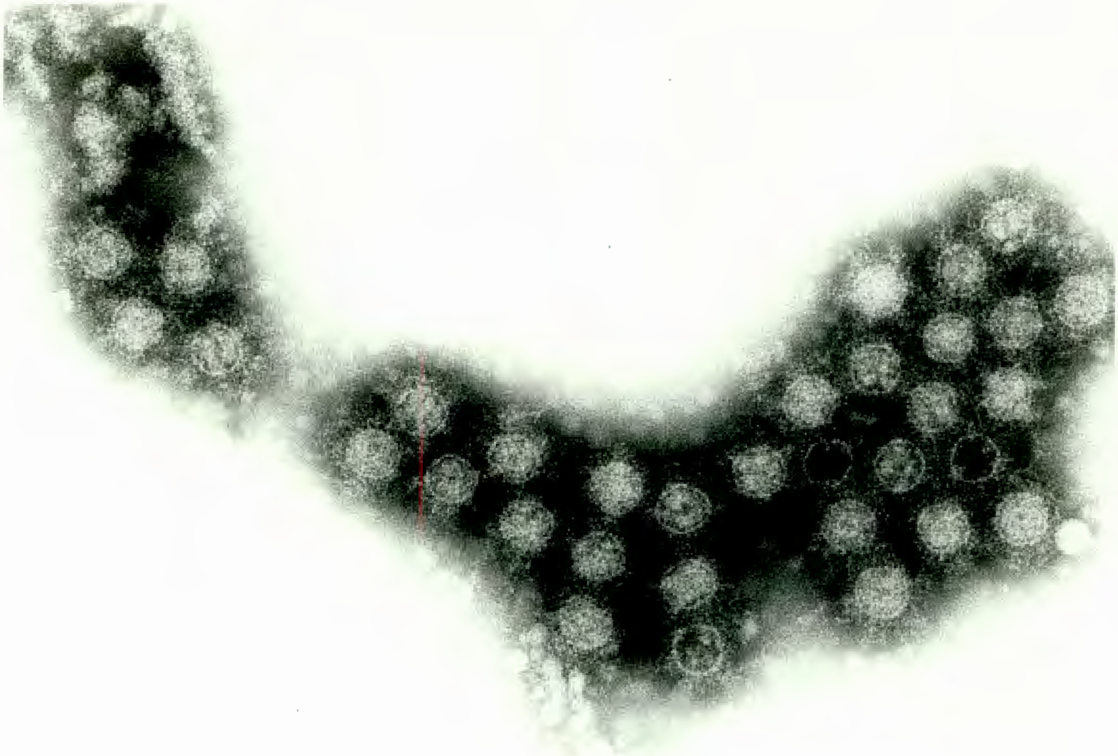
The reaction between test antigen and its homologous antiserum was revealed by the formation of complexes with virus particles. In contrast, virus particles treated with control serum remained dispersed.

In positive reactions, bridges of antibody between virus particles were visible, but the presence of excess antibody obscured details of the particles (Fig. 4.1). When optimal proportions were used, particles linked by antibody were clearly visible (Fig. 4.2). When a mixture of viruses (NPV and CPV) was treated with antiserum homologous to one of them (Fig. 3.9; 4.3), a positive reaction occurred with only the one virus, indicating that the complexing of particles was specific.

Fig. 4.1 *N. cytherea cytherea* CPV in the presence of excess antibody to CPV of *Malacosoma disstria*. Note that each virus particle is surrounded by antibody molecules (x 120 000).

Fig. 4.2 *H. armigera* CPV$_{A2}$ in the presence of optimal proportions of *B. mori* icosahedral CPV antibody. Virus particles are linked by bridges of antibody (x 120 000).

Fig. 4.3 *H. armigera* CPV$_{A1}$ and NPV in the presence of antibody to CPV particles. Note the clumping of CPV particles in contrast to the dispersed NPV rods, capsids and 'empty' capsids (x 120 000).



1

2

3

The CPV_{A1} and CPV_{A2} strains of *H. armigera* were indistinguisable by this technique and each cross-reacted with both CPV strains of *B. mori*. The CPV of *N. cytherea cytherea* reacted strongly with antibodies to *M. disstria*.

4.2.2 Immune Osmophoresis

After electrophoresis in agar gel, virus and homologous antiserum produced visible precipitin lines. Staining enhanced the visibility of weak precipitates (Fig. 4.3). No reaction was obtained with control rabbit serum.

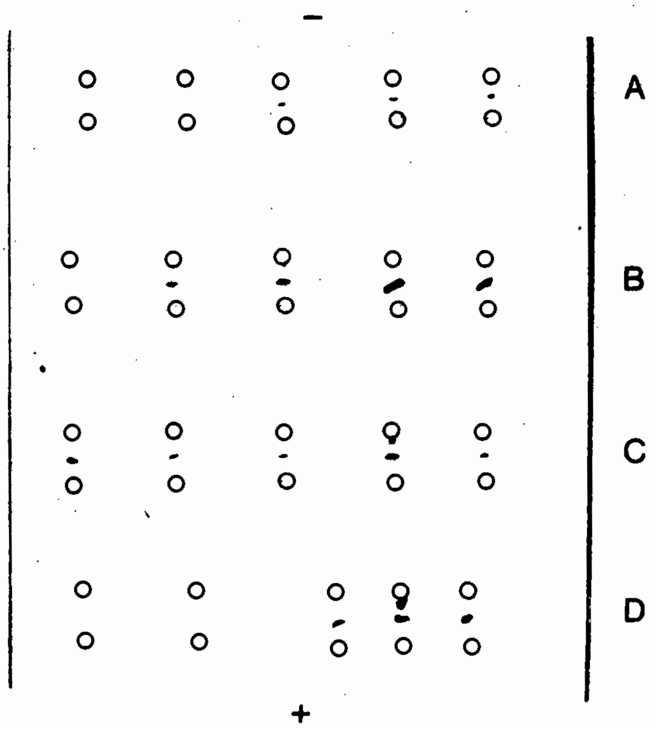


Fig. 4.3 Electrophoresis of sucrose density-gradient fractions of CPV_{A2} against CPV_{A2} antiserum.

Samples in rows:
A, corresponds to bottom of gradient
B, approaches the virus peak concentration
C, after the virus peak
D, approaches the top of the gradient where empty capsids are present

Wells are outlined for clarity.

Antigen-antibody lines of precipitation were observed in the following reactant mixtures:

i) CPV_{A1} and CPV_{A2} of *H. armigera* showed a cross-reaction with their two respective antisera.

ii) CPV_{A2} reacted also with the antisera to the two CPVs of *B. mori* and also to the antiserum to CPV of *M. disstria*. Antigens for *B. mori* and *M. disstria* were not available to test the reverse reactions.

iii) CPV_B of *H. armigera* (Section 8.2.4) cross-reacted with antisera to: CPV_{A2} of *H. armigera*; CPV (icosahedral) of *B. mori*; and, most strongly, CPV of *M. disstria*.

iv) NPV of *H. armigera* showed strong lines of precipitation with the homologous antiserum, and to a lesser degree with GV antiserum.

v) CPV of *N. cytherea cytherea*, like the CPV_B of *H. armigera* reacted strongly with the antiserum to CPV of *M. disstria*.

While these reactions point clearly to a sharing of antigens between the various viruses, the unavailability of some antigens and some antisera leaves the antigenic analysis incomplete.

From the results obtained from the above two techniques (Table 4.1) it appears that:

1) CPV_{A1}, CPV_{A2} and CPV_B of *H. armigera* are antigenically related to one another but not to CPV of *N. cytherea cytherea*;

2) the CPVs of *H. armigera* share some antigens with the two strains of *B. mori* and with that of *M. disstria*;

3) the CPV of *N. cytherea cytherea* shares antigens with the CPV of *M. disstria* but not with that of *B. mori*.

4) there is some sharing of antigens between NPV and GV of *H. armigera* whereas NPV of *H. armigera* is serologically unrelated to CPV of *H. armigera*.

TABLE 4.1

SUMMARY OF SEROLOGICAL INTERACTIONS OBSERVED USING THE TECHNIQUES OF IMMUNE OSMOPHORESIS (IO) AND IMMUNE ELECTRON MICROSCOPY (IE)

Antisera	Antigens									
	H. armigera								*N. cytherea cytherea*	
	CPV_{A1}		CPV_{A2}		CPV_B		NPV		CPV	
	IE	IO	IE	IO	IE	IO	IE	IO	IE	IO
H. armigera:										
CPV_{A1}	++	++	++	++			-	-	-	-
CPV_{A2}	++	++	++	++		+	-	-	-	-
NPV	-	-	-	-		-	++	++		
GV				-				+		
B. mori:										
CPV - icosahedral	+		+	+		+			-	
CPV - hexahedral	+		+	+					-	
M. disstria:										
CPV				+		++			++	++

++ = Strong reaction

\+ = Weak reaction

\- = Negative reaction

Where no symbol occurs, no test was performed.

4.3 DISCUSSION

Classification of ds RNA viruses has in the past been based on serological typing. The serotypes of three of the reoviruses, for instance, were differentiated by their antigenic differences (Sabin, 1959) and the results confirmed by biochemical studies (Shatkin *et al.*, 1968). Because insect viruses are not as host specific as previously believed, serological tests are of great importance for identification. This was stressed in a review of the subject by Mazzone and Tignor (1976). The serological techniques of immune electron microscopy and immune osmophoresis have been used in this study for the detection and identification of viruses.

The standard techniques of serology used in insect virology have been reviewed by Mazzone and Tignor (1976) and include complement fixation, agar gel diffusion, and haemagglutination. The size of intact baculoviruses and CPV viruses makes it impossible for them to diffuse in agar or in cellulose acetate and prevents the use of standard immunodiffusion techniques. Both NPV and CPV of *H. armigera* agglutinated chick red cells but the technique of haemagglutination was not suitable for routine use because of the lack of specificity of the technique. In contrast, the techniques used in this study were highly suitable for the antigenic analysis of these insect viruses. Practical applications of the technique of immune electron microscopy to the rapid diagnosis of certain diseases was reviewed by Kapikian (1975); the technique was sensitive, and low concentrations of infectious agents in the patients convalescent serum could be detected and identified.

The CPV viruses studied here can be divided into two groups according to their serological relationships.

One group consists of the CPV viruses of *H. armigera* and the CPVs from the icosahedral and hexahedral polyhedra of *B. mori*. The second group includes the CPVs from *N. cytherea cytherea* and *M. disstria*, which were found to be serologically related to each other but not to members of the first group, though a weak reaction was observed between CPV_{A2} of *H. armigera* and *M. disstria*. The observed serological relationship between the CPVs of *H. armigera* and *B. mori* indicates the presence of a common antigen or antigens. A study by Hukuhara and Hashimoto (1966) showed a close serological relationship between the strains producing icosahedral and hexahedral polyhedra of CPV of *B. mori*. It would be of interest to ascertain which antigens are shared by the CPV of *H. armigera*.

CPV virus particles from seven different isolates, viz. the CPVs from *Aglais urticae*, *Nymphalis io*, *Arctia caja*, *Phalera bucephala*, *Porthetria dispar*, *Euproctes chrysorrhea* and *Vanessa cardui*, were serologically related to each other but, as suggested by the authors, not to that of *B. mori* (Cunningham and Longworth, 1968). When the serological properties of the polyhedral proteins of some of these viruses, viz. of *A. urticae*, *N. io*, *A. caja*, *P. bucephala*, *A. seleni*, and *P. meticulosa*, were examined, it was confirmed that *B. mori* CPV (Oxford type) was serologically distinct (Payne, 1971). Krywienczyk *et al.* (1969) showed that viruses from *M. disstria* and *Orygia leucostigma* were closely related and shared at least five antigens but only one with CPV of *B. mori*. Krywienczyk and Hayashi (1970) found that this antigen was also shared with midgut ribosomes from both healthy and CPV-infected silkworm larvae. In a further study they attempted to identify the common antigen and found that the ribosomes from a number of sources shared at least one common antigen with free virions of *B. mori* (Krywienczyk and Hayashi, 1971). These authors suggested that the ribose phosphate backbone of RNA was the common antigen.

In subsequent studies, Ikegami and Francki (1973) and Payne and Kalmakoff (1974) found that ds RNA stimulates antibody production and that cross reaction occurs between ds RNA viruses because of the antibody-ds RNA complexes.

The cross-reaction of CPV from *B. mori* with that of another virus, CPV of *H. armigera*, demonstrated in the present work, is the first observation of a strong relationship between the CPV of *B. mori* (Japanese type) and another CPV virus. Despite the presence of a ds RNA component in all the CPV particles tested, no discernible relationship was apparent between the CPV of *N. cytherea cytherea* and either that of *H. armigera* or that of *B. mori*. In the present study, the reason that no cross-reaction was apparent may be that only complete virions were used as antigens. This may have prevented the formation of antibody to ds RNA or the participation of such antibodies in any cross-reactions.

The RNA genomes of CPVs analysed to date have all shown ten segments except for those of *M. disstria* and *A. caja*, which were shown to have sixteen (Hayashi and Krywienczyk, 1972) and twelve (Payne, 1976) segments, respectively. Payne (1976) showed that the CPV of *A. caja* consisted of a naturally occurring mixture of two virus types, those from *N. io* and *Spodoptera exempta*. The CPV of *M. disstria* is probably also a mixture of viruses. If this is the case, it is difficult to evaluate the cross-reaction between antiserum to CPV of *M. disstria* and the CPV of *N. cytherea cytherea* and CPV_B of *H. armigera*, and the weak cross-reaction between the antiserum to CPV of *M. disstria* and CPV of *H. armigera*. There may be a serological relationship with one or both of the presumed viruses.

The finding of Cunningham (1968) that a GV was serologically related to a number of NPV viruses was confirmed in the present study for the NPV and GV of *H. armigera*. Computer analyses on serological cross-reactions and amino acid composition of polyhedrosis virus and GV were made by Bellet (1969), who concluded that the GV and NPV should be grouped together. Serological distinction between certain NPVs and GVs or their components seems difficult. Although these viruses have not been directly involved in diseases other than those of insects (Ignoffo, 1968) studies such as that of Longworth *et al.* (1973), in which antibodies, that reacted with an insect virus from *Gonometa podocarpi*, were found in domestic and wild animals, indicate a need for further evaluation or serological studies.

CHAPTER 5

SYMPTOMATOLOGY AND BIOASSAYS

5.1 METHODS FOR INFECTIVITY STUDIES

Larvae were routinely infected *per os* with the various viruses. The symptomatology of the individual virus infections was studied, and bioassays were performed in order to quantitate the host response.

Methods

A sample of control larvae reared from surface-sterilized eggs was examined for the presence of virus by electron microscopy and nucleic acid studies and found to be free of virus.

Virus-free laboratory-reared larvae (5- and 6-day old) were infected with a virus by applying 0.04 ml of the virus suspension evenly to the surface of the diet; each diet container held one larva. A Bright-Line haemocytometer was used to estimate the number of polyhedral bodies. No counts of samples containing only virions were made, but when virions were obtained by alkali digestion of polyhedra the polyhedral counts are given. However these do not indicate the actual number of virions.

Virus suspensions used ranged from 10^6 to 10^8 polyhedral inclusion bodies (PIB)/ml for CPV; 10^5 to 10^7 PIB/ml for NPV; and 1.8×10^5 particles/g for GV.

The appearance of the larvae, time of pupation, counts of adult survivors, and mortality checks were observed daily at approximately the same time. Extracts of dead larvae or pupae were examined with the light microscope for the presence of polyhedra or capsules and with the electron microscope for the nature of virion. Midguts of CPV-infected larvae were dissected and homogenates were examined in the same way. Some CPV-infected larvae were allowed to pupate and emerge. These were kept for observation.

In assays for viral infectivity, ten-fold dilutions were made from the stock suspensions and added to the medium as described. Where appropriate, further dilutions were made in subsequent experiments. Replicates varied from 5 to 20 larvae per treatment but 6 to 8 larvae per treatment was the usual number. Daily checks were performed to examine the larvae. Assays were ended on death of larvae, or on pupation in the case of NPV-infected larvae, and immediately prior to pupation for CPV-infected larvae. Polyhedral counts were made in the case of CPV-infected larvae where mortality was not a feature of disease. In a similar study, polyhedra of NPV-infected larvae that had died or pupated were counted and correlated with the infecting dose. In addition, infection with the original field mixture of NPV and CPV and infection with laboratory mixtures of these two viruses were compared with assays of single infections.

5.2 RESULTS: SYMPTOMATOLOGY

Insect viruses may be identified on the basis of their physical, chemical and morphological properties as well as by antigenic structure and host specificity (Ignoffo, 1964).

The symptoms observed with the different virus infections were:

5.2.1 Nuclear Polyhedrosis Virus Infection of *H. armigera*

Larvae infected with NPV died 4 to 7 days after ingestion of food inoculated with virus. Their symptoms were typical of NPV infection as described by Smith (1967). Immediately before dying, the larvae became less active and often moved towards the top of the tube from which they hung down in a characteristic limp manner. The insects hung down from the pro-legs and the integument became shiny in appearance. The posterior part of the larvae became distended and starting there, the entire body liquefied. The larvae became flaccid and darkened, and death then followed rapidly. The dead larvae were very fragile and the skin was easily ruptured, liberating a brown fluid that contained polyhedra and virions.

5.2.2 Granulosis Virus Infection of *H. armigera*

A prominent feature of GV-infected *H. armigera* was a colour change. The normally grey, pink, and green larvae became progressively lighter in colour. Many larvae showed an initial increase in size after infection. The larval stage was prolonged, sometimes two-fold, i.e. up to 26 days as compared with an average of 13 days for control larvae. The integument appeared shiny, as if stretched, but remained intact. Thirty-three per cent of larvae did not pupate. Before death, several larvae moved towards the top of the tube in a way similar to NPV-infected larvae. Microscopy of the cream-like body contents showed high concentrations of GV. The original sample of GV contained small numbers of NPV (5 to 10%) relative to GV, and larvae that died 6 to 7 days after infection showed signs typical of NPV-infection. Microscopy of body contents of larvae and pupae that survived, showed GV and only an occasional polyhedron.

Fig. 5.1 For comparison

(a) uninfected control *H. armigera*
(b) CPV-infected *H. armigera*

The larvae were the same age. The bristly appearance of an infected larva is evident.

5.2.3 Cytoplasmic Polyhedrosis Virus Infection of *H. armigera*

Cytoplasmic polyhedrosis virus infections do not usually kill *Heliothis* (Mery and Dulmage, 1975), but if larval stages are infected, the reproductive ability of adults may be affected (Neilson, 1965). The majority of larvae fed on food with added CPV, became infected as determined by the presence of polyhedra. No CPV were found in control larvae. Despite the presence of high concentrations of CPV, mortality was not in excess of that in control larvae. Larvae infected with CPV showed no specific characteristic changes apart from gradual loss of appetite and reduced body size of some larvae; these appeared more bristly, presumably because of the smaller body volume (Fig. 5.1). Most larvae continued to feed normally and appeared similar to control larvae, but the colour of others did become lighter. When larvae were not killed for experimental purposes, they matured, pupated, emerged as adults and laid eggs. The average time to pupation was not prolonged. Uninfected pupae were generally slightly heavier than pupae from larvae infected with CPV and an analysis of the results using Students t-test showed that the difference between virus-infected and control pupae was significant; $P < 0.001$ for CPV_{A2} and $P < 0.005$ for CPV_B.

TABLE 5.1

A COMPARISON OF THE PUPAL WEIGHTS OF UNINFECTED AND INFECTED CPV *H. armigera*

Virus	Weight of control pupae (mg)	Weight of pupae infected with CPV (mg)	Weight of infected pupae as % control (%)
CPV_{A2}†	319 ± 32 (42)	298 ± 29 (38)	93
CPV_B *	319 ± 36 (20)	285 ± 32 (20)	89

( ) = Number of test pupae
† = Infective dose for CPV_{A2} = 1.35×10^6 PIB/larva
* Infective dose for CPV_B = 9.6×10^3 PIB/larva

A few infected moths had crumpled wings and were slightly smaller than normal, but they were fertile and produced a normal quantity of eggs that hatched as fast as control eggs. Light microscopy of the meconium of infected moths revealed both bacteria and inclusion bodies and CPV virions were present in 1-day-old larvae that emerged from unsterilized eggs.

Larvae infected with a mixture of NPV and CPV showed features characteristic of NPV infection and usually died, although the mortality was delayed compared with larvae infected with an equivalent quantity of NPV alone.

5.3 RESULTS: BIOASSAYS

5.3.1 Nuclear Polyhedrosis Virus

The results of bioassays for NPV-infected *H. armigera* larvae including LT_{50} values (the latent time taken for 50% mortality to occur) for a range of infective dosage, are listed in Table 5.2.

TABLE 5.2

MORTALITY OF *H. armigera* LARVAE AFTER INGESTION OF NPV

Number of larvae tested	Infective dose (polyhedra/larva)	Yield of polyhedra/ larva	Total mortality (%)	LT_{50} (days)
a 5	2.3×10^6		100	4-5
8	2.3×10^5		63	5-6
7	2.3×10^4		14	-
5	2.3×10^3		0	-
b 9	2.3×10^6	7.2×10^9	100	4-5
9	2.3×10^5	10.5×10^9	100	4-5
9	2.3×10^4	10.4×10^9	100	5-6
6	2.3×10^3	3.4×10^9	100	5-6
9	2.3×10^2		100	6-7
8	2.3×10	2.4×10^9	88	6-7
7	2.3	2.1×10^9	17	-
5	0.23	1.1×10^9	0	-

NOTE: *a* and *b* were tested at different times of the year and the infective doses in *a* were virions obtained by digestion of the stated number of polyhedra, with subsequent dilutions of virions. Spontaneous mortality in control larvae ranged from 0 to 2%.

Where no figures occur, tests were not done.

Time-versus-mortality curves for a range of NPV-infected dosages fed to larvae is shown in Fig. 5.2. The curves are typically sigmoid and illustrate the increase in mortality with increasing dosage. All the larvae given the five highest infective dosages died, but the time to total mortality was longer in those given the highest infective dose (2.3×10^6 PIB/larva), than in the other four groups. The minimum time to death was four days at 26^0C, and the usual time, five days (Fig. 5.2). The lowest administered virus concentration to cause death was 2.3 PIB/ larva. There is an inverse correlation between infective dose and LT_{50}, i.e. the lower the dose, the higher the LT_{50} (Table 5.2).

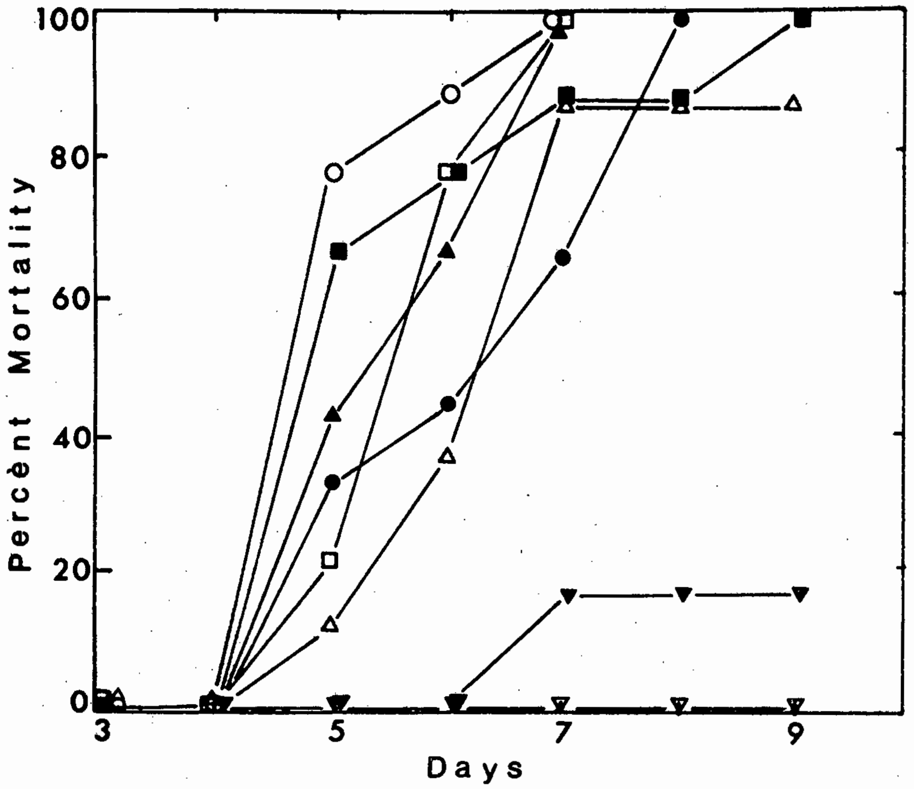


Fig. 5.2 Time-mortality curves for *H. armigera* larvae treated with different concentrations of NPV.

■ = 2.3×10^6 PIB/larva
○ = 2.3×10^5 " "
□ = 2.3×10^4 " "
▲ = 2.3×10^3 " "

● = 2.3×10^2 PIB/larva
△ = 2.3×10 " "
▼ = 2.3 " "
▽ = 0.23 " "

9 larvae for each dilution.

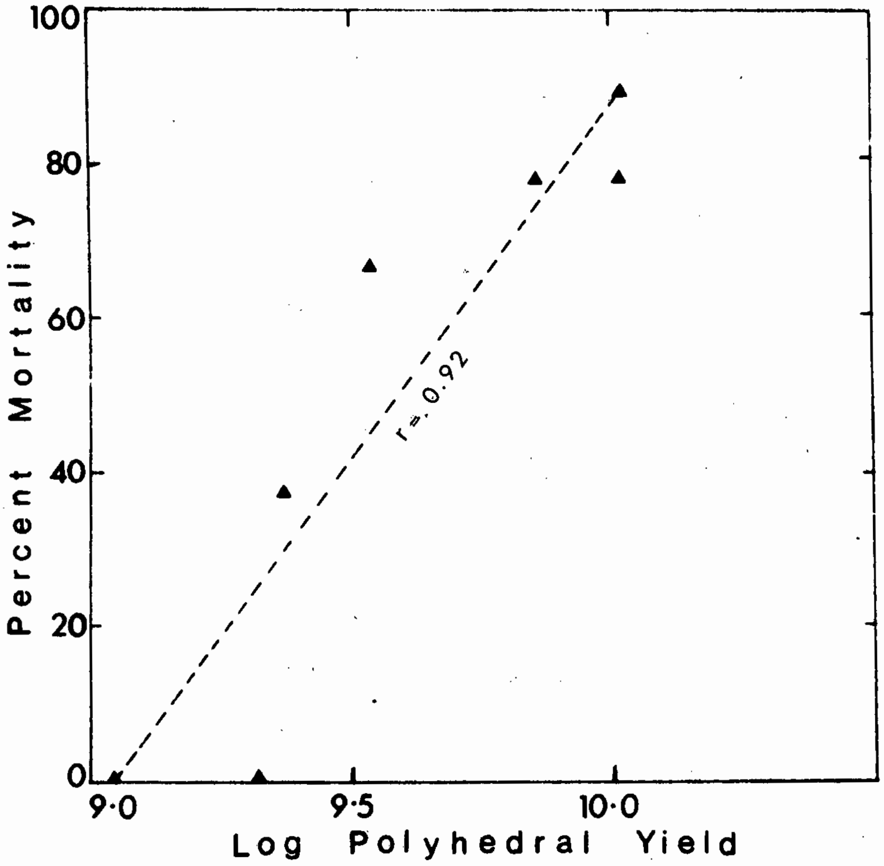


Fig. 5.3 Correlation between mortality of *H. armigera* larvae 6 days after infection with NPV (this is prior to total mortality for the highest infective administered dose) and the yield of polyhedra at the end of the experiment.

A plot of mortality, at six days after infection, versus yield of polyhedra for NPV-infected larvae showed a significant correlation (Fig. 5.3). No increase in mortality, or in yield of polyhedra, was obtained by increasing the dose above 2.3 × 10^4 PIB/larva.

5.3.2 Mixtures of NPV and CPV

Time-versus-mortality curves for larvae infected with mixtures of NPV and CPV are shown in Figs. 5.4 to 5.7. The effect of NPV alone is included for comparison in each case. Total mortality was remarkably similar, in natural mixtures of NPV and CPV where NPV predominated, compared to mortality by NPV alone (Fig. 5.4). Dilution of the virus suspension resulted in typical sigmoid curves for each dosage administered and reduced the mortality, as did dilution of NPV alone. In larvae infected with a 2 : 1 laboratory mixture of CPV and NPV, total mortality was lower than in NPV-infected larvae (Fig. 5.5). In experiments where the CPV dose was constant and the NPV dose varied, the dilution curves followed the sigmoid curve characteristic of NPV with only slightly lower total mortality (Fig. 5.6). However, when the NPV infective dose was constant and the CPV dose varied up to 100-fold, the mortality curves were unaltered (Fig. 5.7).

5.3.3 Cytoplasmic Polyhedrosis Virus

Cytoplasmic polyhedrosis virus of *H. armigera* does not kill infected insects, therefore the criterion used to assess infectivity in this study was the amount of virus, either free or occluded within polyhedra, recovered from infected larvae.

A plot of the relationship between the dose of CPV fed to larvae and the yield of polyhedra recovered from infected larvae six days after infection, is shown in Fig. 5.8. There was no significant difference in the yield of CPV polyhedra after a plateau had been reached, despite increases of the infective dose. The relationship between log dose and the detectable free virus followed a similar trend in each case,

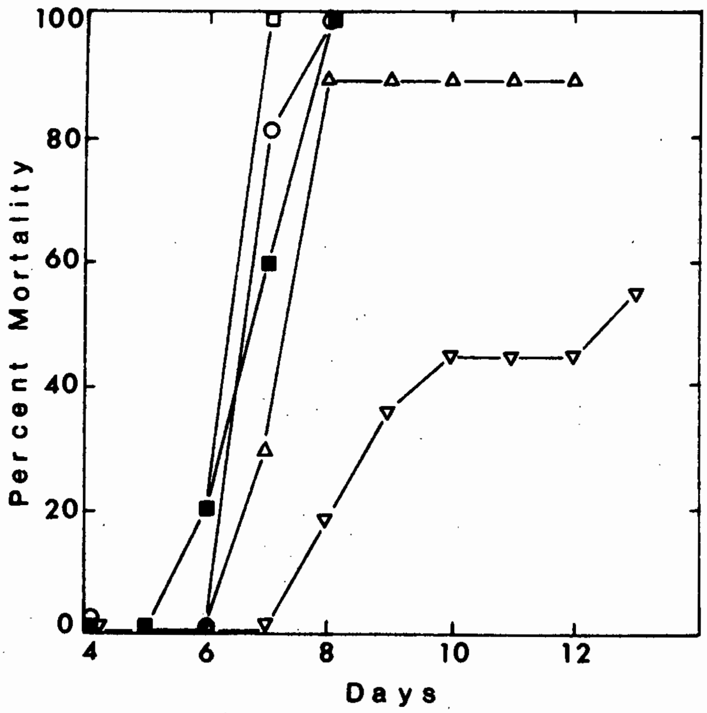


Fig. 5.4 Natural mixtures of NPV and CPV obtained from field isolates. NPV predominates.

□ = 2.3 x 10^8 PIB/larva
○ = 2.3 x 10^7 " "
△ = 2.3 x 10^6 " "
▽ = 2.3 x 10^5 " "
■ = 2.6 x 10^6 NPV PIB/larva only (control)

10 larvae for each dilution

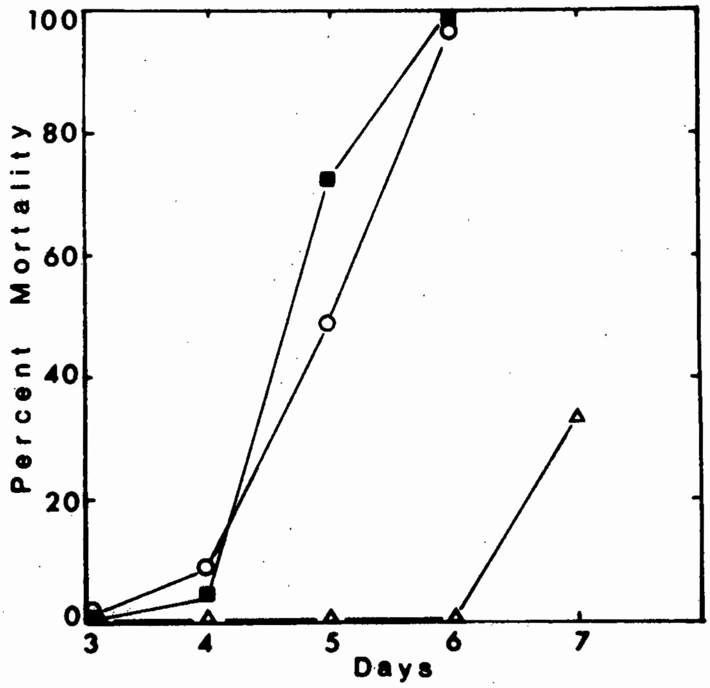


Fig. 5.5 Comparison of effects of NPV alone, mixture of NPV and CPV, and laboratory mixture of NPV and CPV containing an excess of CPV.

○ = 8.6 x 10^6 PIB/larva (natural mixture)
△ = < 2.6 x 10^6 NPV, 5.8 x 10^6 CPV } PIB/larva (artificial mixture)
■ = 3.38 x 10^5 NPV PIB/larva only (control)

25 larvae for each dilution.

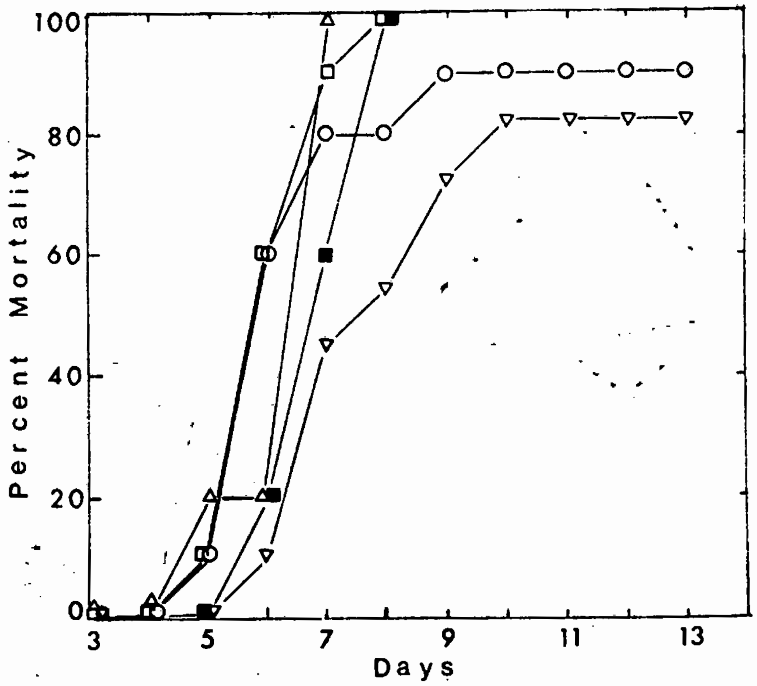


Fig. 5.6 *H. armigera* larvae infected with a laboratory mixture of NPV and CPV. The CPV dose remained constant at 1.5 x 10^6 PIB/larva while that of the NPV varied:

△ = 2.8 x 10^8 PIB/larva
□ = 2.8 x 10^7 " "
○ = 2.8 x 10^6 " "
▽ = 2.8 x 10^5 " "
■ = 2.8 x 10^6 NPV PIB/larva only (control)

10 larvae for each dilution.

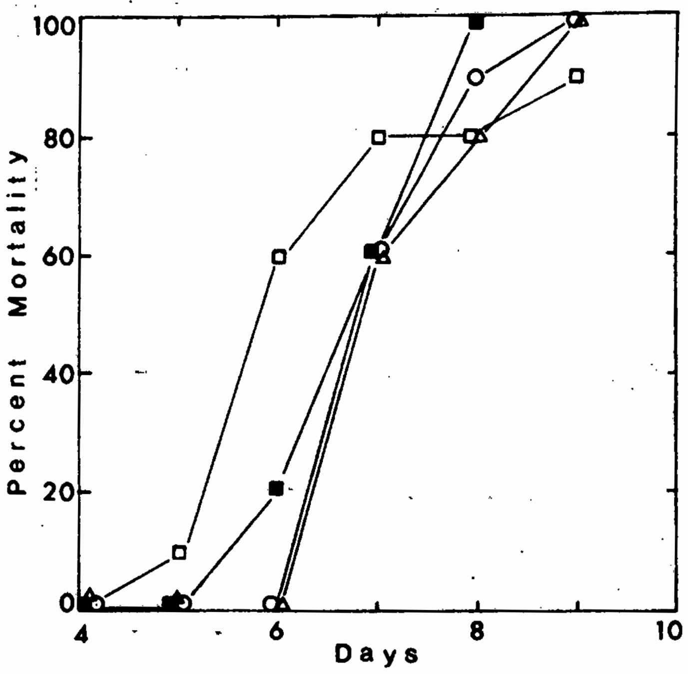


Fig. 5.7 *H. armigera* larvae infected with a laboratory mixture of CPV and NPV, NPV being kept constant at 2.8 x 10^6 PIB/larva.

○ = 1.5 x 10^7 CPV PIB/larva
□ = 1.5 x 10^6 " " "
△ = 1.5 x 10^5 " " "
■ = 2.8 x 10^6 NPV PIB/larva only (control)

10 larvae for each dilution.

5.3.4 Variation in Polyhedral Size with Length of the Infection

Light microscopy examination of samples of CPV polyhedra 2 to 10 days after infection revealed the presence of many more large polyhedra in samples from 5 days onwards after infection. The polyhedral suspensions were washed with distilled water and centrifuged, and the pelleted material was examined in the electron microscope to study the size increase of polyhedra. The dimensions of polyhedra were measured on the electron micrographs (Fig. 5.9) taking the widest diameter of each polyhedron. The mean size and variance are shown in Table 5.3. Measurements for NPV polyhedra are included for comparison.

TABLE 5.3

RELATIONSHIP BETWEEN SIZE OF POLYHEDRA AND TIME AFTER INFECTION

Virus	Days after infection	Size			Number counted
		Range (nm)	Mean (nm)	S.D. (nm)	
CPV	3	1200 - 1840	1520	320	19
	5	1040 - 2550	1800	760	12
	7	1110 - 3020	2060	950	29
	10	1750 - 3610	2680	930	19
NPV	5-6	830 - 1360	1090	260	30

The polyhedral size increased progressively with time. The size range of NPV polyhedra was restricted to the lower range of CPV polyhedra up to day 7, corresponding to the initial period of CPV infection which is the time that NPV-infected larvae often die. Examination of the polyhedra on the micrographs revealed the presence of CPV particles on the surface of the smaller, immature CPV polyhedra but not on large CPV polyhedra nor on NPV polyhedra (Fig. 5.9).

In the later stages of CPV infections, the polyhedra appeared in bundles or bunches, as if enclosed by a membrane (Fig. 3.1), and were difficult to separate for counting. No such bundles were seen either early in CPV infections or in NPV infections.

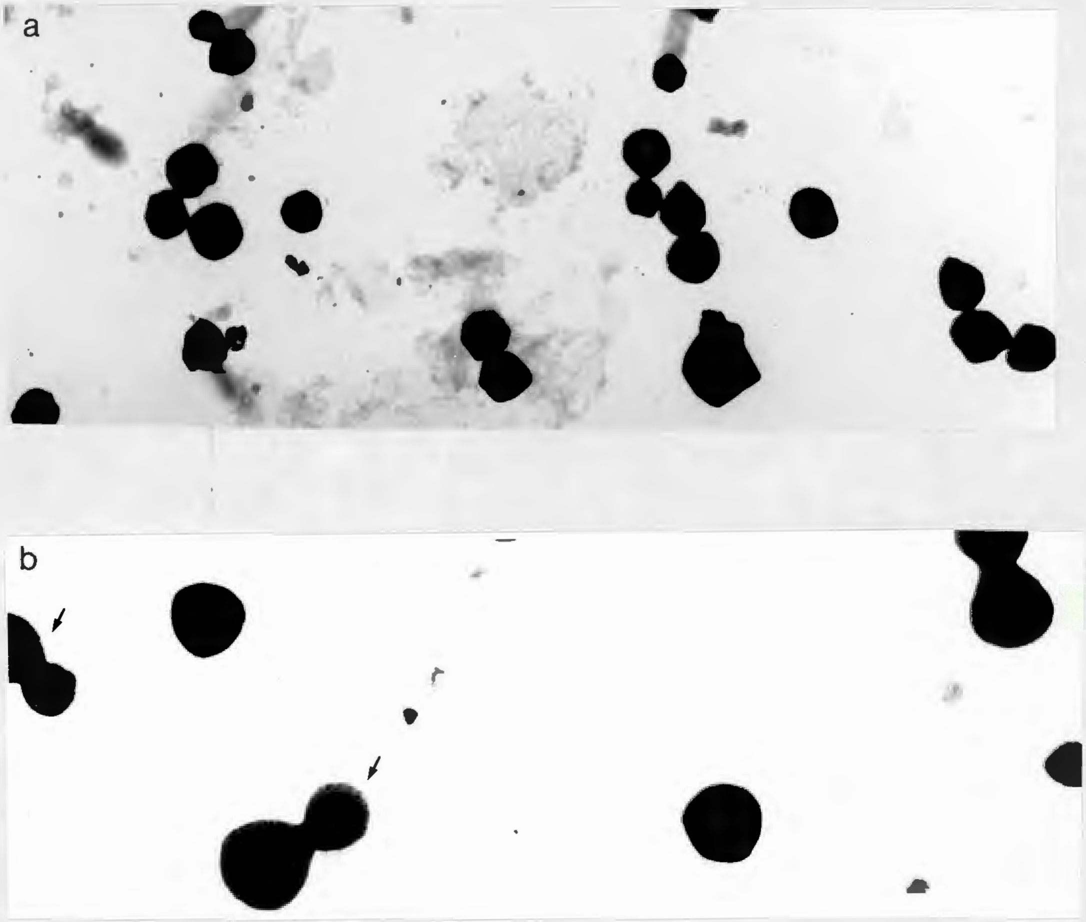


Fig. 5.9 Electron micrographs of polyhedra.

(a) NPV polyhedra. They are irregular in shape (x 6000).

(b) CPV polyhedra 3 days after infection. The shapes are more regular than those of NPV. Some globular polyhedra have virus particles (arrowed) at the surface (x 6000).

Fig. 5.9 Electron micrographs of polyhedra.

(c) CPV polyhedra 5 days after infection. A variety of polyhedral shapes are seen in increasing size. Surface particles are still present on small polyhedra (x 6000).

(d) CPV polyhedra 7 days after infection. Note the increased size of certain polyhedra, on which virus particles are not evident. The shapes of the polyhedra are more clearly defined; six-sided polyhedra appear common. These correspond to the icosahedral shape described by Hukuhara and Hashimoto (1966) and the rhombic dodecahedron and dodecahedron and cube described by Rao (1973) (x 6000).

(e) Polyhedra 10 days after infection. Many large polyhedra are seen (x 6000).

5.4 DISCUSSION

Symptomatology

The symptoms displayed by *H. armigera* larvae after infection with NPV, GV, and CPV were different for each virus.

The symptoms of *H. armigera* infected with NPV are similar to those described by Whitlock (1974), who was able to distinguish between NPV- and GV-induced infection of *H. armigera* larvae on the basis of the symptoms displayed. The integument of NPV-infected *H. armigera* larvae in the present study was shiny, like that described by Smith (1967). The whitish colour of *Pectinophora gossypiella* larvae infected with NPV (Vail *et al.*, 1972) was not seen in the present study.

In *H. armigera* infected with a mixture of NPV and either GV or CPV, the dominant features were characteristic of NPV infection. In *H. armigera* larvae that were infected with both GV and NPV but that had only a minimal number of NPV polyhedra present, symptoms were similar to those described by Whitlock (1974) for GV-infected larvae. Prominent characteristics were a lightening in colour and size increase six days after infection. The size increase of some larvae may be directly correlated with increase in the amount of fat body tissues, in which viral replication proceeds. The prominence of infected fat body tissue was readily visible in histological sections (Fig. 6.20). Death of the larva was generally later in GV than in NPV infections. Breakdown of the integument as described by Smith (1967) in cutworms was not apparent.

An synergistic enzyme present in the capsules of *Trichoplusia ni* GV has been shown to have a synergistic action with NPV in mixed infections (Tanada *et al.*, 1973). A similar synergistic factor with enzyme properties was isolated from the GV capsule infecting *Pseudaletia unipuncta* and enhanced infection with an NPV in the same host (Tanada *et al.*, 1975). A synergistic factor in the capsules of GV infecting *H. armigera* may enhance a concomitant NPV infection; this would account for the early death of GV-infected larvae in which a few NPV polyhedra were present.

The current understanding of the endocrine processes underlying insect growth and development has been recently reviewed (Judy, 1974). There are three hormones involved:

(1) the brain hormone;
(2) ecdysone, produced by prothoracic glands in the thorax; and
(3) the juvenile hormone (JH) secreted by the corpora allata.

The latter hormone directs the qualitative expression of each moult. When a relatively high concentration of JH is present in the insect's haemolymph the ensuing moult will be larval in nature, whereas when JH is absent the succeeding moult will be towards the adult. The JH is a lipid and may be bound to a water-soluble carrier for transport in the haemolymph. Whitmore and Gilbert (1972) have demonstrated binding of JH to a lipoprotein of high density. In the third instar, protein synthesis in fat body is normally reduced. Therefore if the metabolism of the fat body is altered in any way, as by the presence of replicating viral material, the continued stimulation of JH may be induced, or the balance between JH and ecdysone could be altered. The extension of the larval period (the number of instars has not been reported) and absence of pupation in some GV-infected larvae may be due to interference of normal hormonal changes in the fat body tissues. The effect of viral replication itself within fat body cells may alter the amount of glycerides formed, resulting in depletion of food stores. The presence of a minimal quantity of stored food may be necessary to stimulate the pupal moult. Because GV-infected larvae feed longer and die later than NPV-infected larvae, GV may not prove as effective as a biological control agent. On the other hand, survival of GV capsules because of resistance to digestion, and their survival intact when ingested by predators such as the egret (Gitay and Polson, 1971) will contribute to the continued spread of the disease.

With current interest in the potential use of viruses as factors for biological control of insects, most work has been restricted to the lethal effects of the viruses. CPV does not cause marked mortality but does affect the size and fecundity of surviving adults (Neilson, 1965). In the present study, *H. armigera* larvae infected with CPV showed no external signs of disease and the mortality was not higher than in control larvae.

Bioassays

LARVAE INFECTED WITH NPV:

Time-versus-mortality plots for a range of infective NPV dosages showed a correlation between increased viral dose and mortality. When dilutions of viruses as opposed to polyhedra, were fed, there was a more direct response because the number of virions enclosed within polyhedra vary. The disease manifests itself in approximately four days, regardless of the administered dose. The LT_{50} values, on the other hand, increased in relation to decreasing dose. These observations are similar to those of Whitlock (1974).

The steepness of the response increased with increasing concentration of virus administered; larval mortality varied directly with dosage. Whitlock (1974) found that a concentration of 800 PIB/ml was the lowest dose that caused death, whereas 60 PIB/ml (2.3 PIB per larva) was the lowest dose in the present study to cause mortality.

The present study shows that *H. armigera* larvae are highly susceptible to NPV. A comparison of the effects of NPV, GV, and CPV on *H. armigera* indicate that, of the three, NPV is the most suitable potential candidate for use as a biological insecticide. This is further emphasized by its short incubation period, with corresponding shorter time of feeding and higher mortality. In addition, the positive phototropic reaction displayed by infected larvae would increase their exposure to predators and parasites (Whitlock, 1974). Field trials, such as those conducted by Roome (1975) in extension of laboratory tests, confirm that NPV of *H. armigera* is suitable for control of the bollworm.

For its use as a biological control agent, large quantities of the virus would be required. The highest yield of NPV has to date been obtained by infecting the natural host, i.e. from larvae fed on an artificial diet. Ignoffo obtained a 2000- to 5000-fold (1965a) and 5000- to 10 000-fold (1973) increase in the number of PIBs from each diseased *Heliothis* larva. Alternative means of growing insect viruses have been tested. Wells (1970) attempted to replicate insect viruses in certain microorganisms such as bacteria and yeasts using fermentation procedures and reported a 400-fold increase in viral replication in *H. zea*. On the other hand, our experiments did not confirm these findings (Rubinstein and Polson, 1976 - Appendix 1). A quantitative *in vitro* study by Raghow and Grace (1974) indicated that the maximum increase of *B. mori* NPV virions grown in Grace's *B. mori* cell line was approximately 25 times (i.e. input 8.9×10^4 $LD_{50}/10^6$ cells with maximum titre of 2.2×10^6 $LD_{50}/10^6$ cells). In a recent study by Vaughn (1976), NPV was produced in large-volume cell cultures. For an input of 5 ml of 10^5 -10^6 Pfu/ml the maximum yield was 6.38 × 10^7 PIB/3-5 × 10^6 cells. This is an increased yield but neither these yields nor those of Wells (1970) were comparable to those reported by Ignoffo in larvae. In the present study the increase in polyhedral yield for NPV of *H. armigera* larvae was found to range from 3 × 10^3 - 10^9 PIB/larva. To date, the use of larvae to obtain large amounts of virus seems the most suitable means.

LARVAE INFECTED WITH CPV:

The bioassays undertaken with *H. armigera* larvae infected with CPV were more difficult to interpret than those with NPV-infected larvae, as the former gave very low mortalities. Therefore to assess infectivity the amount of virus produced in infected larva was monitored either by counting polyhedra or by detecting free virus at the end of experiments. In an early study in which the yield of CPV polyhedra was correlated with infecting dose, there was a direct relationship between dose and polyhedral yield up to a certain level, beyond which the yield decreased. The results may indicate interference. A similar trend was observed by Magnoler (1974) in studies on the CPV of *P. dispar*. This author found that the highest mortality resulted from infection with the second-lowest dose and that the highest dose of virus resulted in the lowest mortality; Magnoler suspected an interference phenomenon due to a mixed virus infection. In the present study, no virus particles or viral nucleic acids were found in aliquots of control *H. armigera* larvae from the same larval stock as those used for CPV infectivity studies. However this does not rule out the stimulation of latent virus production as a result of infection with another virus. Subsequent experiments in which the polyhedral yield of *H. armigera* larvae was examined for a range of CPV dosages showed no significant difference in polyhedral yield. All larvae treated with the virus became infected but the yield was not related to the dosage. This finding is also in agreement with that of Magnoler (1974).

This erratic relationship between the amount of virus administered and yield of polyhedra found for CPV is similar to the correlation between dosage and mortality observed in *H. armigera* larvae infected with GV (Whitlock, 1974). Whitlock could not establish a defined relationship between dose and mortality. A study of CPV-infected *H. armigera* larvae in which the polyhedral yield was correlated with length of infection showed a direct increase in the number of polyhedra until a constant number of PIB/larva was reached. This may indicate a limiting factor due to exhaustion of nutrient supplies or the metabolic activity of the host could be affected with development of the virus in midgut cells. Alternatively midgut cells when full of polyhedra may slough off and eventually be excreted, thus stabilizing the number of polyhedra.

A marked decrease in free amino acids in the haemolymph and midgut of *B. mori* following CPV infection was reported by Kawase (1965), and in addition Kawase and Hayashi (1965) showed that hypoproteinemia of virus-infected insects was a result of polyhedra or virus formation in the midgut combined with the starvation effect due to midgut malfunctioning. On the other hand, Watanabe (1970) found that ^{14}C glycine incorporation into all protein fractions was greater in diseased than in healthy midguts throughout the viral infection period. He suggested that CPV infection might induce active synthesis of midgut protein in addition to polyhedral protein. The infection of *H. armigera* larvae with as few as 6 to 8 CPV polyhedra/larva in the present study suggests that defective particles did not constitute a significant proportion of the total virus population.

The observation that the number of CPV polyhedra produced remained static at a certain level whereas the average size increased with time suggests that increased viral production is related to incorporation within maturing polyhedra.

Observations in this study confirm the conclusion of Aruga and Israngkul (1961) that polyhedra get larger with time. In some species such as *B. mori*, polyhedra tend to be large (Smith, 1967), whereas in *Antheraea pernyi* they may be both very large and very small (Smith, [redacted] 1963). The presence of both small and large polyhedra throughout CPV infection of *H. armigera* indicates that both types occur in such infections. Smaller polyhedra are possibly immature and may increase by deposition of polyhedral protein and incorporation of virus particles. In addition virus particles are seen on the surface of the small CPV polyhedra, which lack an obvious membrane, whereas in the large polyhedra, a membrane or definite outline of the polyhedra is visible and no virus particles are seen. This observation was also noted in thin sections of midgut cells, in which a wide range of CPV polyhedra was present in the same cell (Fig. 6.17). In *H. armigera* the membrane in CPV polyhedra is only visible in a mature polyhedron and at a later stage than that of NPV polyhedra. No virus particles were seen on the surface of NPV polyhedra of *H. armigera* and the polyhedral outline was well defined as if membrane-bound.

The size of NPV and CPV polyhedra were similar at 3 to 7 days after infection and may explain the difficulty in separating the two types of polyhedra in mixed CPV and NPV infections. Larvae in mixed infections are killed early by NPV (4 to 6 days after infection), at which stage the two viral types have similar-sized polyhedra. The volume frequency distribution of samples of NPV from the same insect species is unimodal (Martignoni and Iwai, 1968, cited by Martignoni *et al.*, 1969), whereas that of CPV polyhedra is multimodal (Martignoni *et al.*, 1969).

As infection progressed in *H. armigera*, bundles or bunches of CPV polyhedra were found. This phenomenon is similar to that described in *A. pernyi*, where polyhedra appear in 'colonies' surrounded by a membrane. A similar phenomenon was also described by Bird and Whalen (1954) in *Choristoneura fumiferana*.

CHAPTER 6

HISTOPATHOLOGICAL STUDIES

The histopathology of virus infections of *Heliothis armigera* was studied by means of light and electron microscopy. Of the various histological methods of fixation tested, such as 10% formalin or 95% ethanol, that of Dubosq-Brasil, described below, gave the best results. Dehydration of material for histology with ethanol was not as satisfactory as treatment with ethanol and butyl alcohol (Stiles, 1934). The modified Azan staining technique of Hamm (1966) stains polyhedra bright red.

6.1 METHODS

6.1.1 Reagents:

Histology

Dubosq-Brasil fixative:

Ethanol-water 80% (v/v)	150 ml
Picric acid	1 g
Formol saline 10% (v/v)	60 ml
Glacial acetic acid	15 ml

Electron microscopy

Palade's fixative:

Osmium tetroxide 2% (*m*/v) in distilled water	5 ml
Veronal-acetate buffer ...	2 ml
Hydrochloric acid, 0.1 M .	2 ml
Distilled water	2 ml

Histology

Butyl alcohol dehydration:

Step	Ethanol in water (%)	(ml)	Butyl + alcohol (ml)	Time (h)
a	35			½
b	45	9	1	2
c	62	8	2	2
d	77	65	35	4
e	90	45	55	6-24
f	90	25	75	6-12
g	Repeat *f*			
h	Butyl alcohol only			
i	Repeat *h*			

Electron Microscopy

Ethanol dehydration:

Step	Ethanol in water (%)	Time (min)
a	35	10
b	70	10
c	96	10
d	absolute	10
		Repeat x 3

Virus-free larvae of *H. armigera* reared in the laboratory were fed on a diet contaminated with purified samples of GV, NPV, or CPV, or with the original field material consisting of a mixture of NPV and CPV. Control larvae not fed with these suspensions were kept under similar conditions. At daily intervals after infection, larvae were killed and fixed either for histological examination with the light microscope, or for ultrastructural studies.

6.1.2 Histological Methods

Larvae were cut in two and placed in Dubosq-Brasil fixative for two to four days. A graded series of butyl alcohol and ethanol mixtures was used for dehydration. The tissues were transferred to a mixture of two parts of paraplast (Sherwood Medical Industries, m.p. 56°C) and one part butyl alcohol and kept covered at 60°C for 24 hours, then uncovered until the smell of butyl alcohol had disappeared. The tissues were then transferred serially to three changes of paraplast, for two hours each, before final embedding.

Sections of 4 to 5µm were cut with a Reichert Sledge microtome and floated onto glass slides coated with 0.5% gelatine and 0.05% chrome alum in order to retain the sections on the slides. Sections were stained with the modified Azan stain of Hamm (1966): after treatment with acetic acid, the sections were stained in the azocarmine solution for 15 min followed by the same period in the counterstain containing aniline blue, orange G, fast green and phosphotungstic acid. After dehydration in ethanol and clearing in toluene, the sections were mounted in DPX (BDH) and examined in a light microscope. In addition, control and GV-infected material were stained with Heidenham's iron haematoxylin and counterstained with erythrosin (Huger, 1961).

6.1.3 Preparation of Thin Sections for Electron Microscopy

Special fixation, embedding, and cutting techniques are necessary to study the ultrastructure of cells in the electron microscope. The method of fixation used combined glutaraldehyde and osmium tetroxide (OsO_4) (Ledbetter and Porter, 1963). It is based on methods developed by Palade (1952), who used OsO_4. This stabilises cellular constituents and enhances definition of structures. The method of Sabatini *et al.* (1963) makes use of aldehyde fixation to preserve subcellular detail more faithfully. The properties necessary for a good embedding medium are low viscosity for good penetration, correct degree of hardness to allow sections as thin as 50 nm to be cut and stability of the medium in the electron microscope. Embedding media such as epoxy resins and propylene oxide have been used: Spurr's epoxy resin (1969) was used in this study.

The resolution obtained in the electron microscope is largely dependent on the nature of the section. Staining increases the contrast in thin sections: uranyl acetate (Epstein and Holt, 1963) and lead citrate (Reynolds, 1963) are widely used.

Method

Pieces of tissue, 1 mm^3 in size, were dissected and fixed for 2 h at 4^{0}C in 5% glutaraldehyde in phosphate buffer pH 7 (PB). Tissues were washed in the same buffer and post-fixed for 1-2 h on ice in Palade's fixative. After washing in buffer, the tissue was dehydrated by immersion in a series of solutions of increasing ethanol concentration. The embedding medium was prepared by adding 10 ml of ERL-4206 (vinyl cyclohexene dioxide), 6 ml of DER-736 (diglycidyl ether of polypropylene glycol), 26 ml of NSA (nonenyl succinic anhydride), and finally 0.4 ml of S-1 (dimethylamino-ethanol) to a dry container using glass syringes. Tissues were placed in 50% embedding medium in ethanol for several hours at room temperature to allow the ethanol to evaporate. They were then transferred to pure embedding medium in dry gelatine capsules and kept at 60^0 for one day.

Blocks were first trimmed to expose the tissue on a trapezoidal face. Thick sections were cut using a Sorvall Porter-Blum ultra-microtome and glass knives made on an LKB knife-maker, type 7801B (Glauert and Philips, 1965). These sections, 1μm thick, were mounted on glass slides. Thin sections were cut with a glass or diamond knife and silver or grey sections (less than 100 nm thick) (Peachey, 1958) were mounted on uncoated copper grids and allowed to dry.

Thick sections were dried and stained by heating on a hot plate with 1% toluidine blue in 1% borax in distilled water, and examined in a light microscope to determine the best area for thin sectioning. Thin sections were stained on a piece of dental wax, initially with 2% (*m*/v) aqueous uranyl acetate solution for 3 min followed by washing in distilled water. After drying, sections were stained with lead citrate (0.1 to 0.4% in distilled water to which 1% 10 M NaOH was added) for 2-3 min in a closed dish containing saturated KOH to absorb CO_2. Sections were washed in distilled water, dried, and then viewed with a Siemens Elmiskop 1A electron microscope.

6.2 RESULTS

Larvae infected with NPV, CPV, and GV were examined by light and electron microscopy to study the intracellular replication of the viruses and their distribution in the tissues. Histological changes resulting from virus infection could then be compared and contrasted. Most of the observations in this study were made in the midgut area because earlier studies (reviewed by Smith, 1963) had shown that this is the major site of CPV replication.

6.2.1 Uninfected Larvae

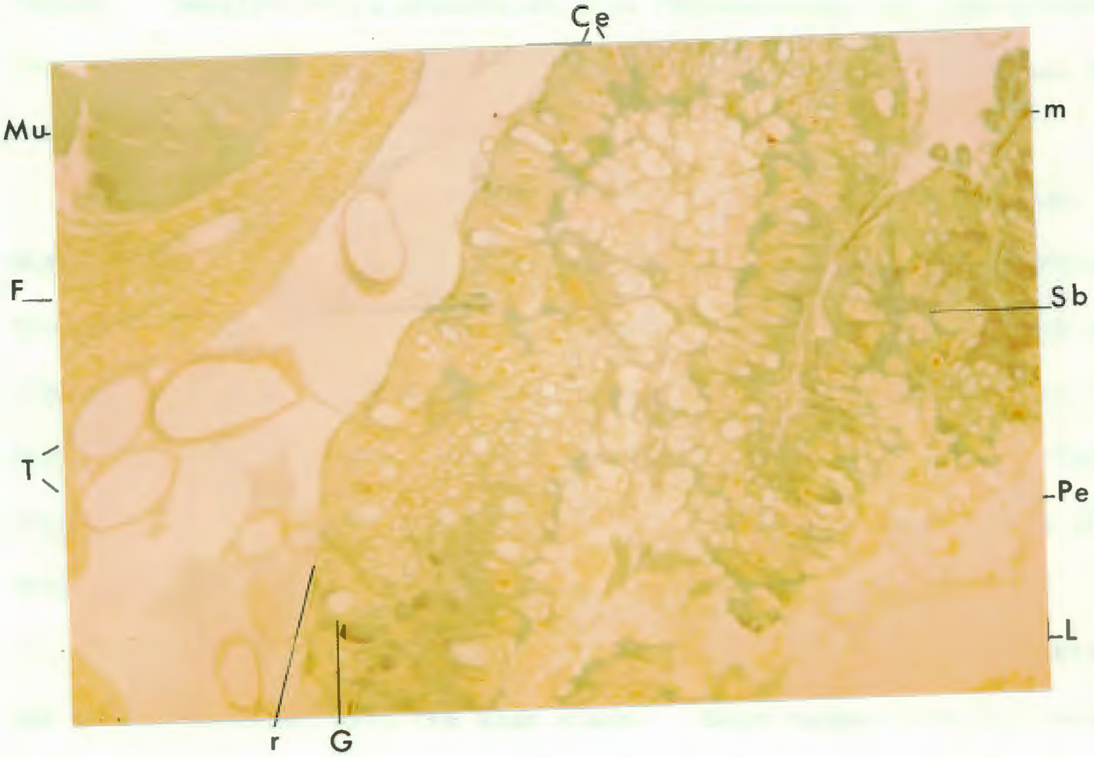
A transverse section of an uninfected larva of *H. armigera* is shown in Fig. 6.1. Around the lumen of the gut the mucosa consists of a thick layer of columnar epithelial cells with a well-defined striated border. The cytoplasm of the columnar epithelial cells looks granular, and the nuclei are centrally situated. The peritrophic membrane is visible in the lumen of the gut (Fig. 6.1).

Flask-like goblet cells are seen between the columnar epithelial cells at irregular intervals, their number varying with their position

Figs. 6.1 and 6.2 Transverse sections of an uninfected larva of *H. armigera* stained with the modified Azan stain. Some of the normal features are indicated. These are:

lumen - (L); midgut columnar epithelial cells - (Ce); goblet cell - (G); regenerative cell - (r); muscle - (Mu); sub-epithelial muscle fibres - (m); fat body cells - (F); tracheole - (T); malpighian tubule - (Mt); periotrophic membrane - (Pe); striated border - (Sb); hypodermis - (H); epicuticle - (E).

Fig. 6.1 (x 400).
Fig. 6.2 (x 200).


Ce
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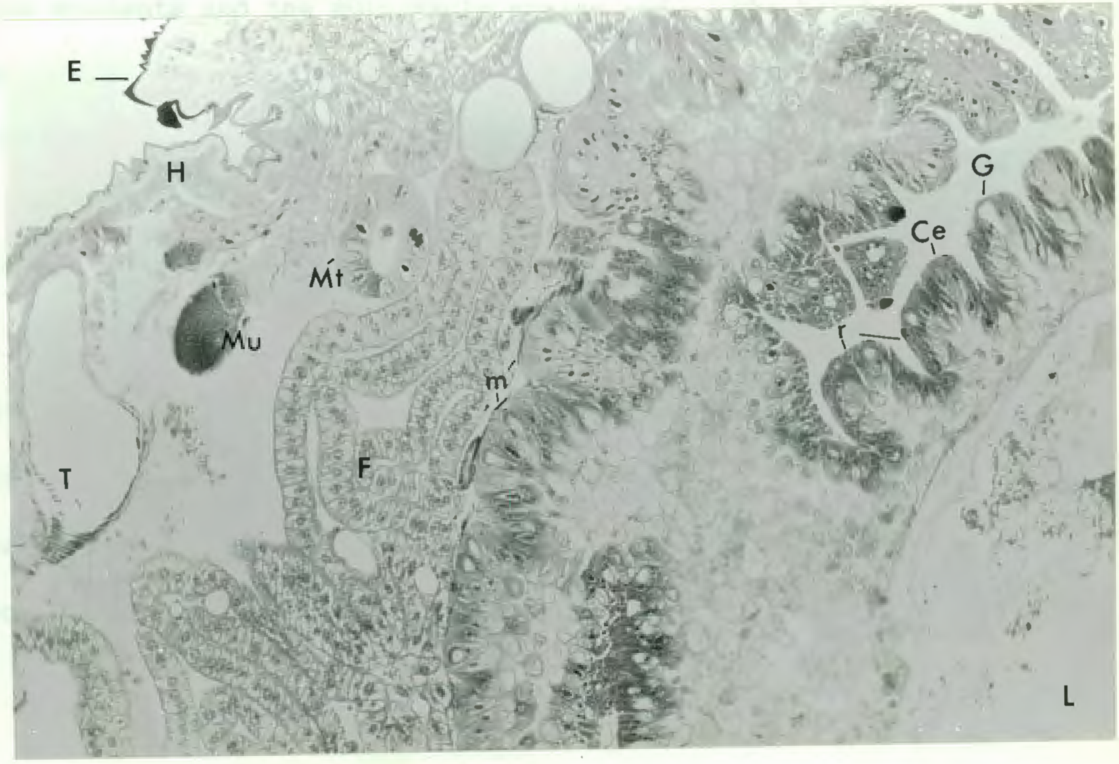
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in the midgut and the larval species. The goblet cell has a large internal cavity with a striated lining; the cavity narrows in the direction of the lumen. The nuclei are usually located in the basal region. Smaller cells associated with regeneration are also present. Two muscle layers are distinguishable, the more bulky longitudinal muscle bundles exterior to the circular muscle layer.

In Fig. 6.1 the fat body cells, closely packed and numerous, are distinctive structures that are separated from the basement membrane by the loose submucosal connective tissues in which there are numbers of tracheoles of varying luminal capacity and irregular outline. In Fig. 6.2 additional structures visible are the malpighian tubules and the hypodermis. Blood cells and silk glands, though not apparent in this photograph, were identified in the histological preparations.

Each of these histological structures is made readily visible by the colour obtained with the Azan stain. This ranges from yellow-green, light blue and pale brown for fat body cells, epidermal cells, muscle, endocuticle and midgut epithelium. Silk glands are green with blue or red contents and the epicuticle stains red. The bright red staining of polyhedra was not seen in the cells of the uninfected larvae of *H. armigera.*

6.2.2 Larvae Infected with NPV

The rust-red staining of the polyhedra was not evident in the first 24 h after infection but became apparent the next day. By the third day there was a most intense specific staining of the polyhedra-infected nuclei of the cells of the fat body, the tracheal matrix, and to a lesser extent the epidermis (Fig. 6.3). In the more advanced

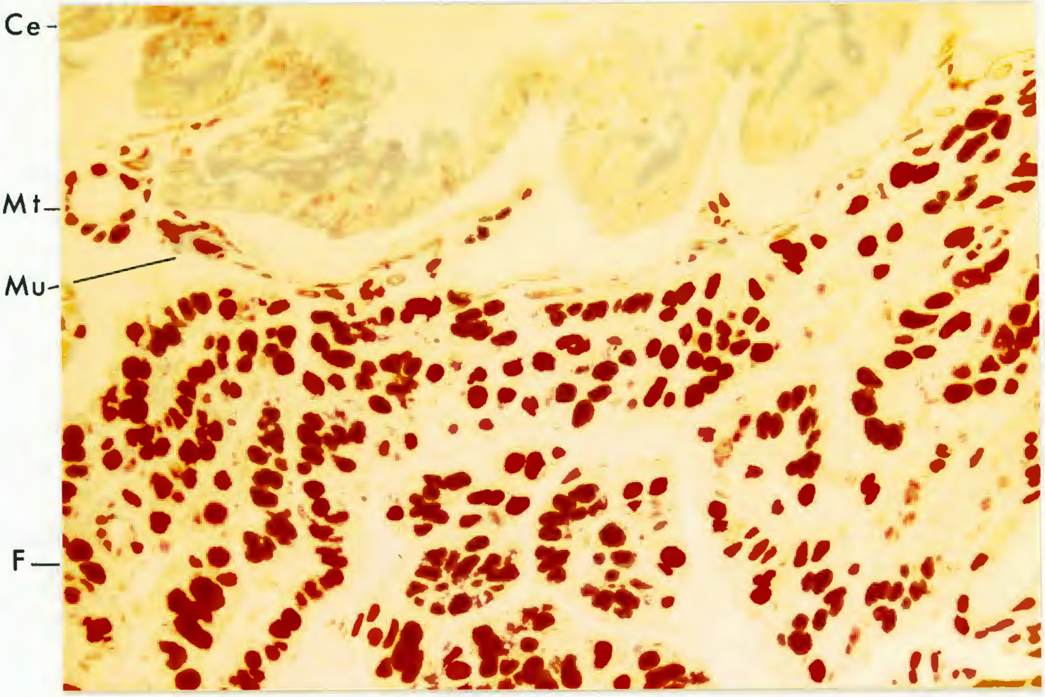


Fig. 6.3 Section through *H. armigera* larva three days after infection with NPV. Note the presence of red-staining polyhedra within nuclei of fat body cells (F), malpighian tubules (Mt), and periphery of muscles (Mu). No polyhedra are visible in the midgut epithelial cells (Ce) (x 400).

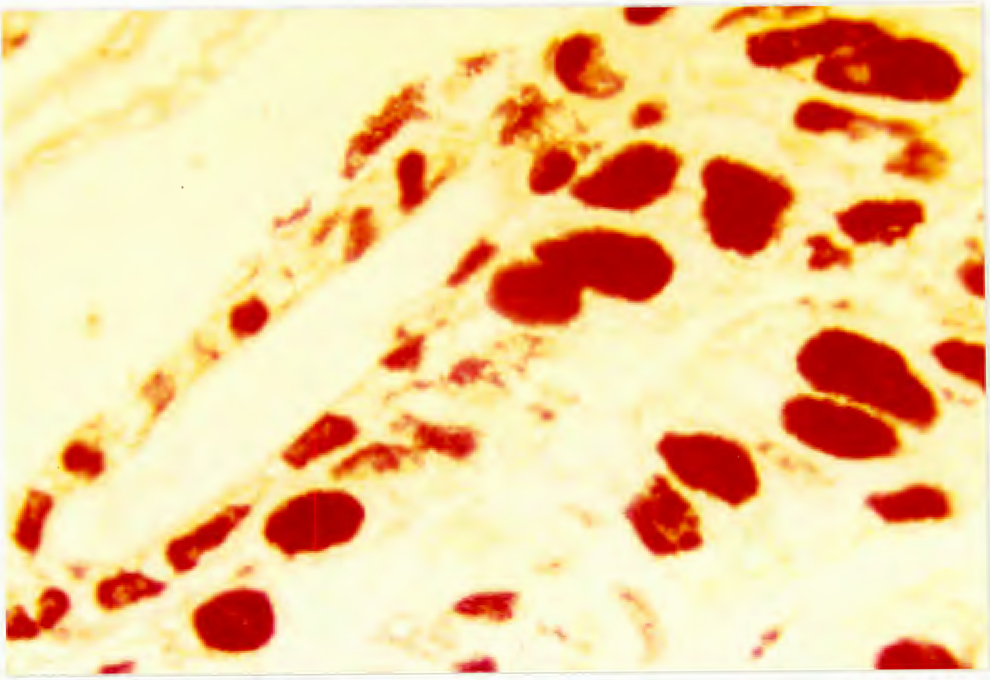
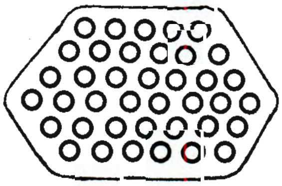


Fig. 6.4 A high-powered view through the fat body and tracheole of *H. armigera* larva five days after infection with NPV. Note the heavily infected nuclei, some of which have become disrupted (x 1500).

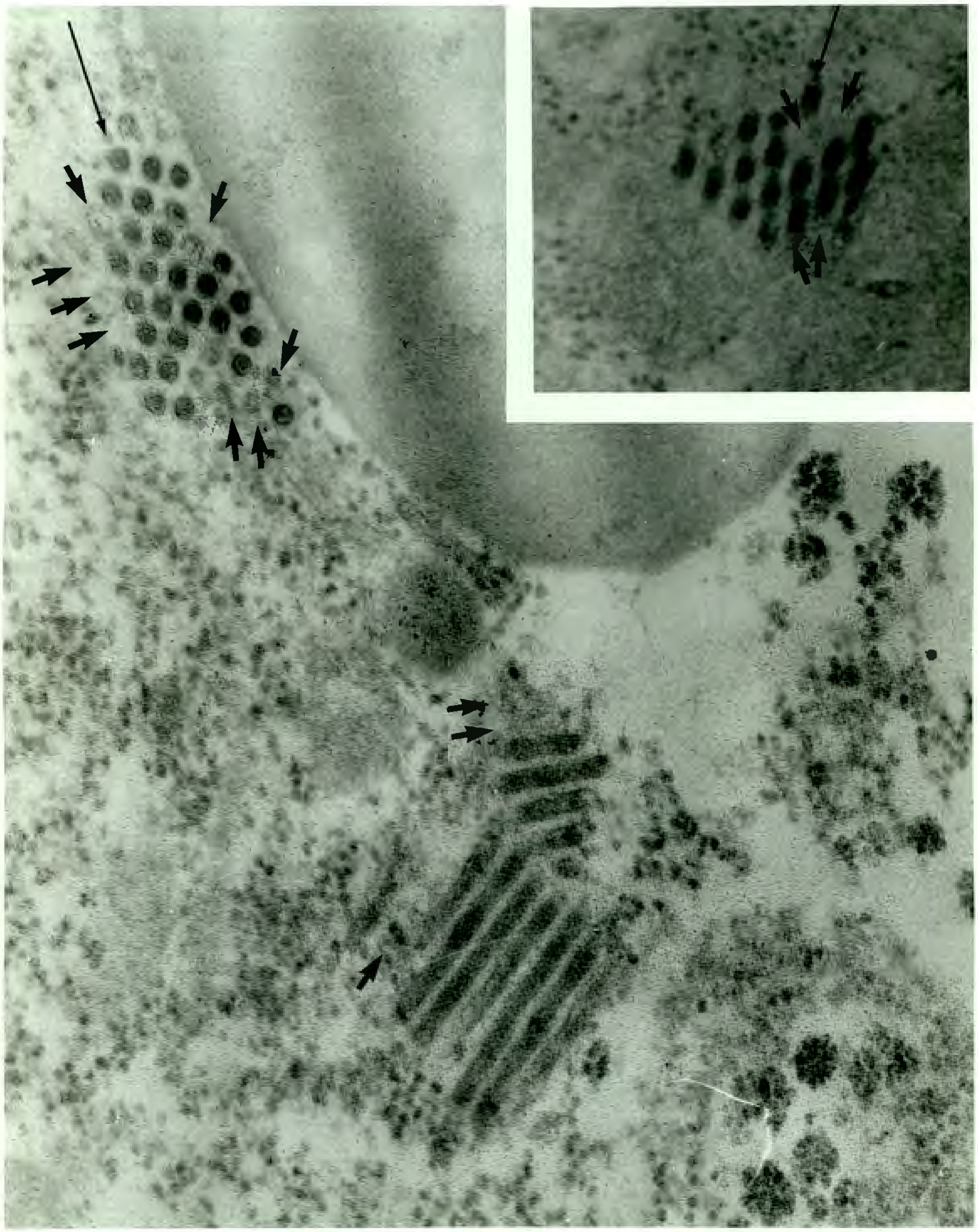
stages of infection (e.g. by day 5) (Fig. 6.4), polyhedral staining was present in most of the tissues including the silk glands, hypodermis (Figs. 6.5 and 6.6), malpighian tubules, blood cells, and sarcolemma of muscle. At higher magnification (× 1500) the granular nature of the staining of the nuclei was more obvious and in many instances the distended nuclei in the cells of the fat body had ruptured, releasing the polyhedra (Fig. 6.4). The mucosa separated from the submucosa, and the epithelial cells became granular and less well defined (Figs. 6.5 and 6.6). The nuclei of the columnar epithelium appear to stain light brown, but this is not due to the presence of polyhedra. The difference in staining is clearly seen in Fig. 6.3, where the bright red of the polyhedra contrasts with the pale brown of the nuclei.

Thin sections of the fat body cells examined by electron microscopy revealed the presence of virus rods seen in longitudinal or transverse section (Fig. 6.7). Although these oriented rods suggested assembly, no formation of polyhedra was seen. Of the sections studied, this photomicrograph best shows the irregular arrangement of the nucleocapsids. The nucleocapsids cut in cross-section suggest a pattern of 39 nucleocapsids. A longitudinal section of the nucleocapsids cut almost parallel to the axis shows eight parallel nucleocapsids, and the beginning of envelope formation is seen in the semi-longitudinal section. In addition a pattern of 22 nucleocapsids is also present. From these sections NPV of *H. armigera* appears to conform to the description "many nucleocapsids per envelope" (Summers, 1975).

Fig. 6.7 Electron micrograph of an ultrathin section through a fat body cell of an NPV-infected *H. armigera* larva five days after infection. The virions are arranged in oriented groups and have been sectioned both longitudinally and transversely. In the longitudinal section, eight parallel rods are seen though two are not easily visible. The arrangement of the virions in cross-section forms a pattern of 39 nucleocapsids as shown diagrammatically. The inset shows 22 nucleocapsids, arranged (1-2-3-4-5-4-3). Thin arrows indicate the central row and thick arrow-heads show hollows of envelopes indicating the lack of nucleocapsids (x 90 000).



39 (5·6·7·8··7·6) (Kawamoto and Asayama, 1975)



6.2.3 Larvae Infected with CPV

The first evidence of specific staining of cytoplasmic polyhedra occurred on the second day after infection. In contrast with what was seen with NPV-infected larvae, the earliest rust-red staining was seen in scattered columnar epithelial cells (Fig. 6.8), starting as discrete cytoplasmic granules at the base of the cells on day 2. The first discrete polyhedra were observed at the base of the few columnar epithelial cells by the following day. By day 5 the cytoplasm had become packed to a varying degree with red polyhedra in the majority of the cells seen in longitudinal or transverse section in any one field, and this packing increased with time (Fig. 6.9). No polyhedra were observed in other tissues. In sections of the midgut prepared from larvae as late as nine days after infection, the light staining fat body (Fig. 6.10) looked similar to the fat body in controls just before pupation, i.e. vacuolation of fat body tissue, with loss of contents during processing. The remaining fat body appeared carminophilic. The intensity of the staining of the cells packed with polyhedra is shown in Fig. 6.11. More detail of the CPV-infected cell is evident in Fig. 6.12.

The subcellular structure of the uninfected columnar epithelial cells is illustrated in Figs. 6.13 - 6.15. The close apposition of cytoplasmic membranes of adjacent cells (Fig. 6.13) is broken only occasionally by minute spaces. Cross-linking between the two membranes is seen as a reflection of the dentate processes of the surfaces. These processes are clearly visible about the villous projections cut in transverse section in Fig. 6.15.

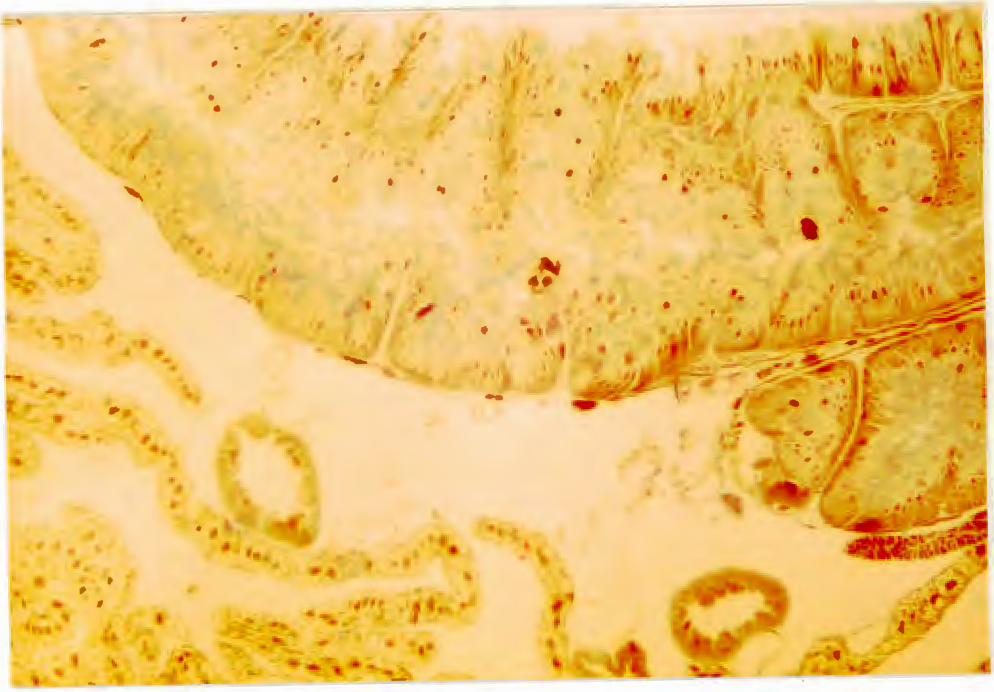


Fig. 6.8 Section through infected larval midgut epithelium of *H. armigera* two days post-infection with CPV. Note the scanty polyhedra in the midgut epithelium and absence of polyhedra in other tissues (x 400).

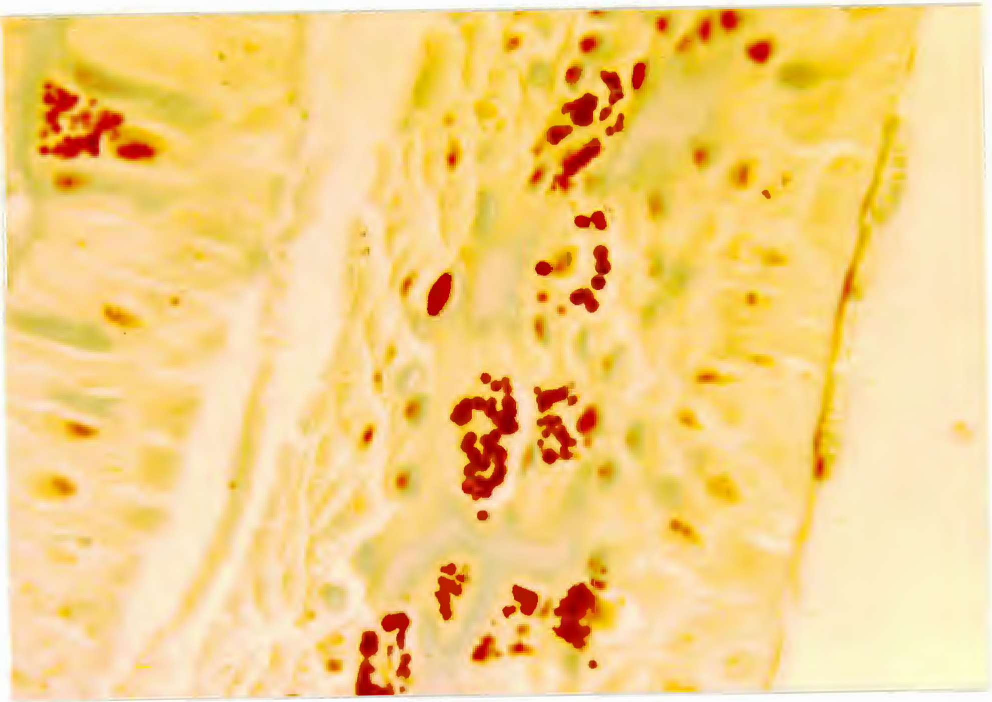


Fig. 6.9 *H. armigera* larva four days after infection with CPV. The polyhedra are situated in the apical zone of columnar epithelium cells. Note the range in size of polyhedra (x 1500).

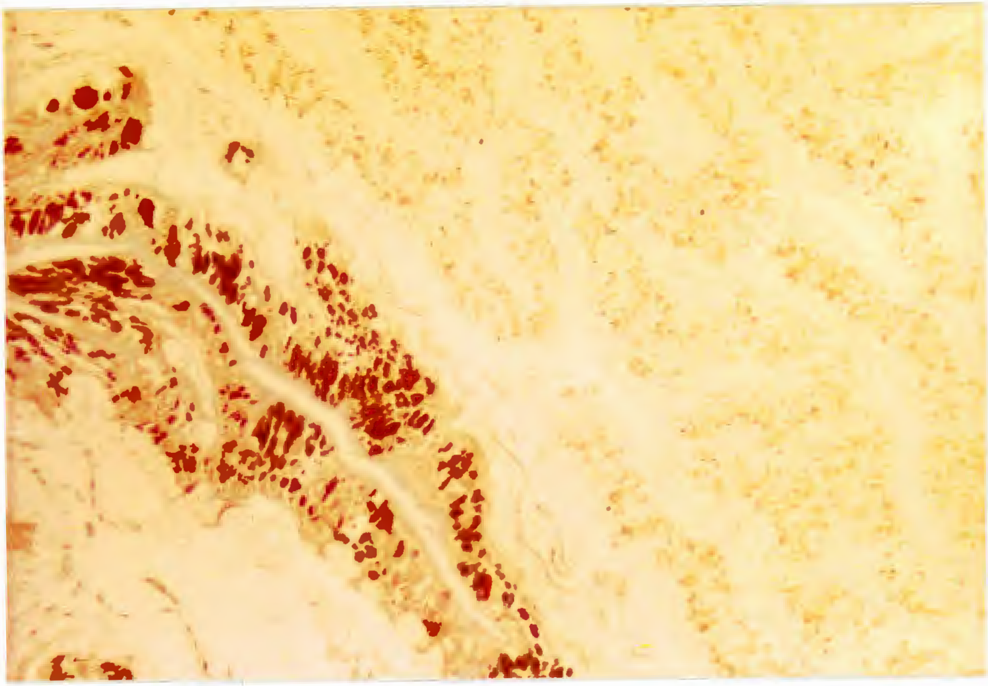


Fig. 6.10 Section through *H. armigera* larva nine days post-infection with CPV. Polyhedra are present in most epithelial cells. Note the pre-pupation appearance of fat body cells (x 400).

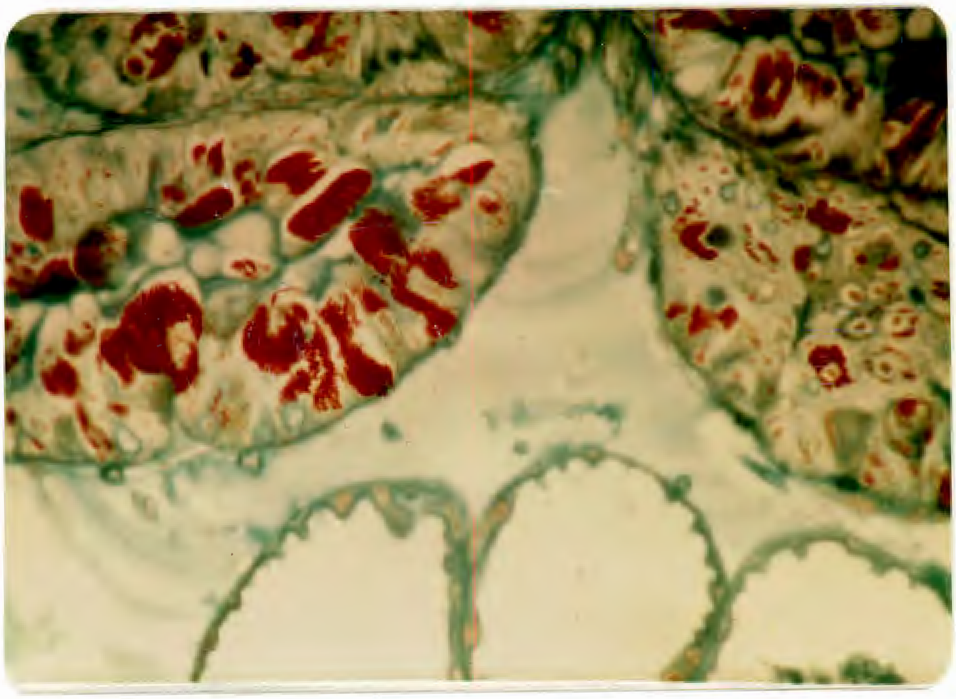


Fig. 6.11 Detail of infected midgut cells of *H. armigera* infected larva (x 500).

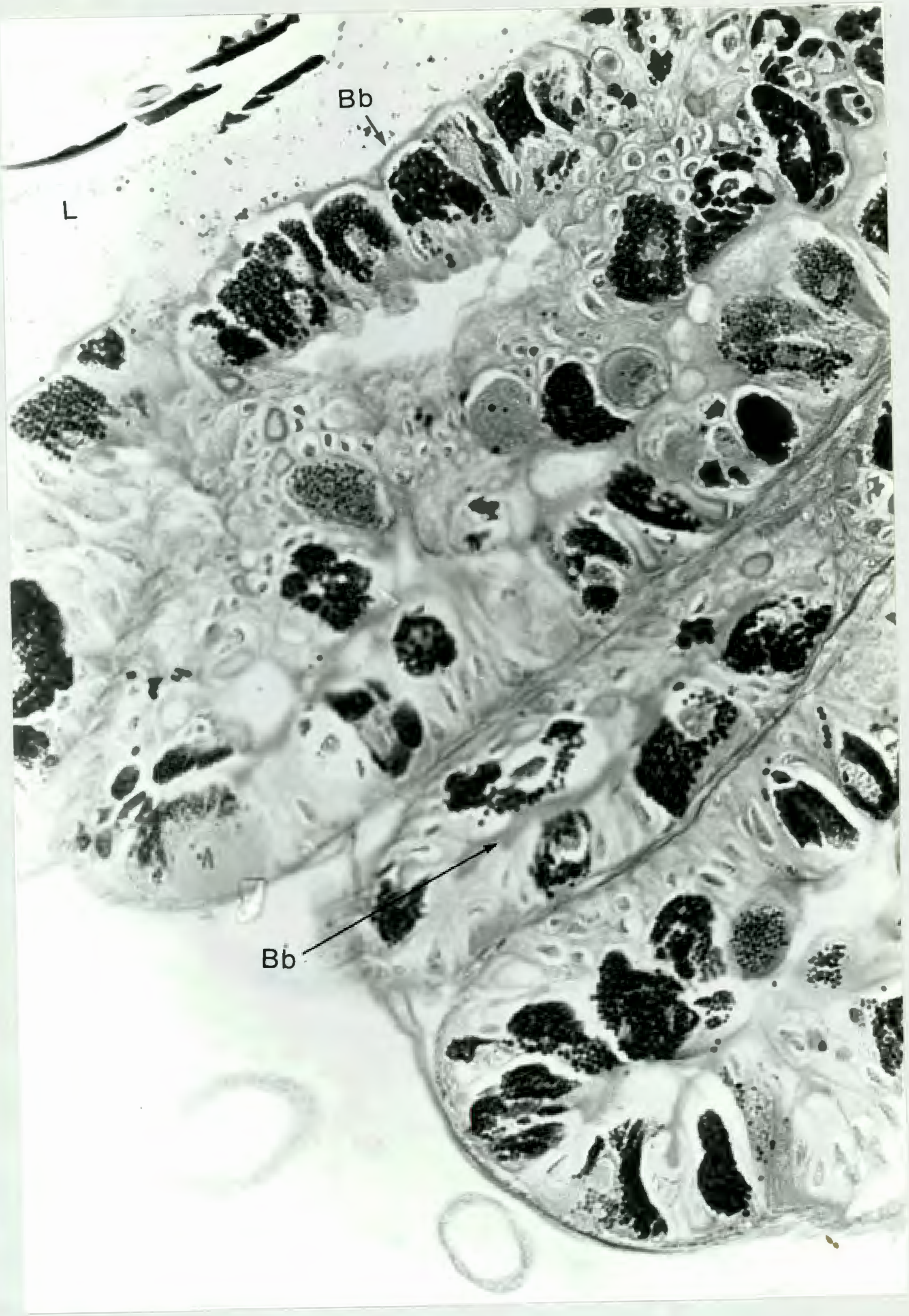


Fig. 6.12 Section through midgut epithelial cells of *H. armigera* infected larva. Note the CPV polyhedra arranged in rows at the base of epithelial cells and the uninfected tracheoles. The brush border (Bb) is clearly seen. Lumen - (L) (x 700).

Beneath the cell membrane are the easily identifiable organelles such as ribosomes, both free and attached to the many short lengths of endoplasmic reticulum; and the mitochondria circular, elongated, dumbbell-shaped or horseshoe-shaped - each with well preserved cristae (Figs. 6.13 and 6.14). The cell nuclei with their surrounding double-layered membrane circumscribe the granular chromosomal material and the more densely packed particulate nucleoli (Figs. 6.13 and 6.14).

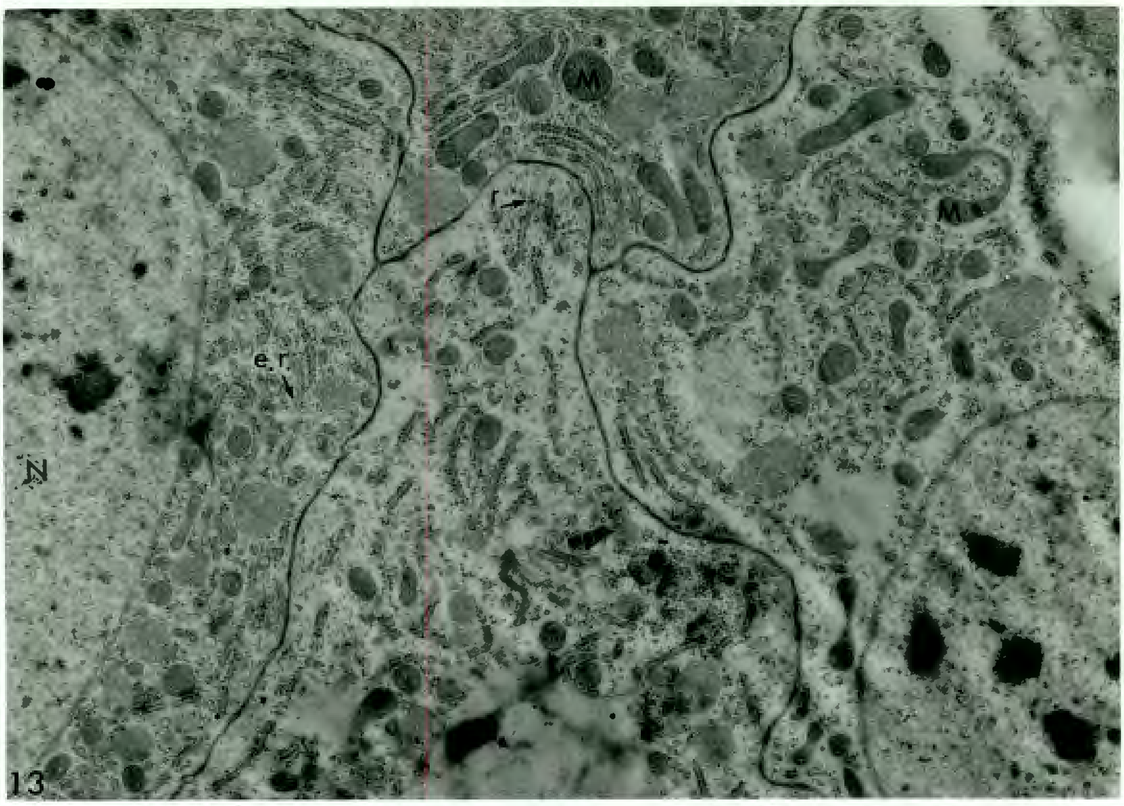
In contrast the CPV-infected epithelial cells in thin sections show many large, darkly staining bodies, the cytoplasmic polyhedra. These are seen as electron-dense particles in a slightly less dense body consisting of spherical or angular masses of "crystallogenic matrix" or stroma (Figs. 6.16 and 6.17). These bodies vary in size and shape, though all exhibit angular sides and are grouped in ill-defined beds of finely granular "viroplasm" (Fig. 6.16). Mature polyhedra appear to have a limiting membrane, in contrast with developing polyhedra. At higher magnification, the nature of the polyhedral matrix can be seen to be crystalline. The virus particles are visible both randomly distributed inside the polyhedra, and scattered in large numbers in the viroplasm. The particles consist of an electron-dense central core surrounded by a less dense capsid (Fig. 6.17), more easily seen in the viroplasm outside the polyhedra. Some particles are seen in the process of incorporation into polyhedra.

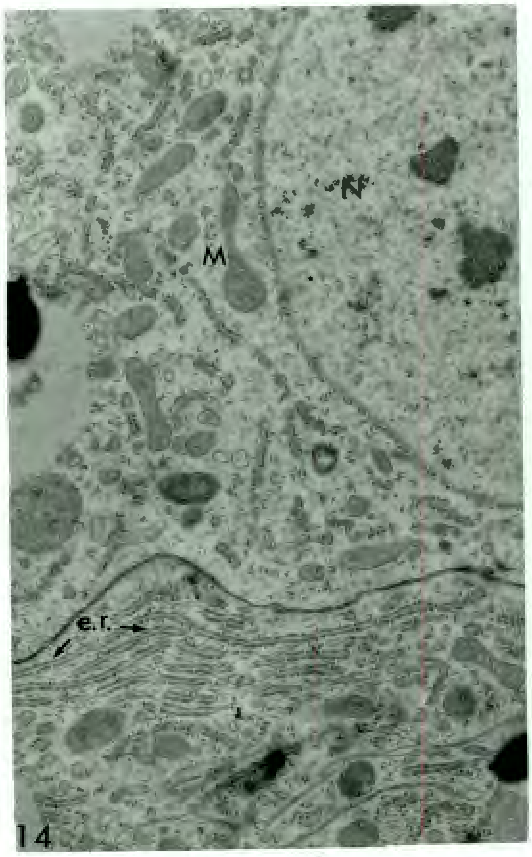
As these complexes increased in number during the period following infection, the organelles of the surrounding cytoplasm became less distinct and degenerated. Mitochondria and endoplasmic reticulum were visible only at the periphery of the cell. There were no virus particles in the nuclei of the infected cells, and nuclei appeared normal.

Fig. 6.13 Thin section through uninfected columnar epithelial cells showing four adjacent cells. The nucleus (N) is enclosed by the nuclear membrane and the chromatin is dispersed in irregular masses. Mitochondria (M) are present in a variety of shapes and the endoplasmic reticulum (e.r.) with ribosomes (r) are seen (x 12 500).

Fig. 6.14 Section through uninfected columnar epithelial cell of *H. armigera* larvae. Note the regularly arranged endoplasmic reticulum (e.r.) and dumb-bell-shaped mitochondria (M). The nuclear membrane surrounding the nucleus (N) is clearly seen (x 15 000).

Fig. 6.15 Transverse section of midgut columnar epithelial cell which includes section through the 'brush border'. Note the apical microvilli which project into the gut lumen. Each projection is covered with a fine coat of minute fibrils, the glycocalyx. Narrow filaments within the cytoplasm are seen as dots (arrowed). The arrowed microvilli (-G) belong to a goblet cell. This micrograph also illustrates the fine structure of intercellular junction in the midgut. The apposed lateral cell membranes are linked at intervals by tight junctions (TJ) and septate desmosomes (SD) (x 24 000).


M
M
e.r.
N
13


N
M
e.r.
14

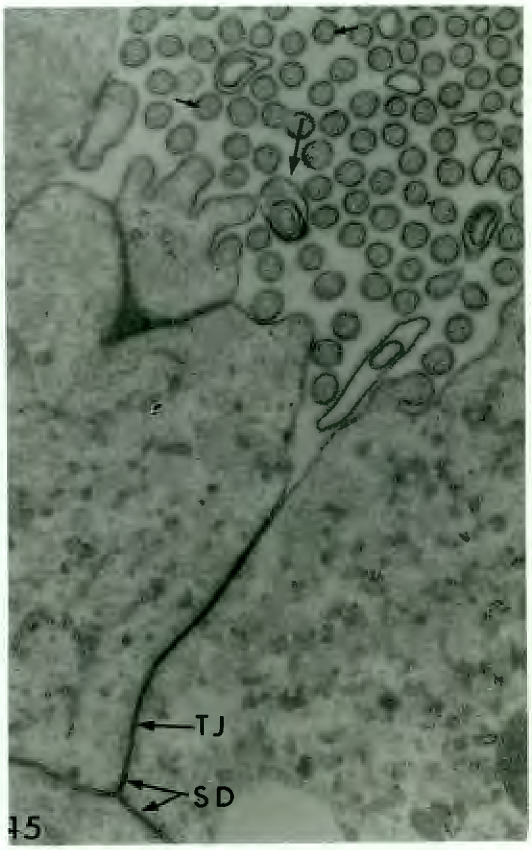

TJ
SD
15

Fig. 6.16 Electron micrograph of thin section through CPV-infected larval midgut epithelial cell. Note polyhedra (P) scattered at random, virus particles (VP) present both within the polyhedra and free in the crystallogenic matrix (CM) and the virogenic stroma (VS) (virus particles are formed in the VS prior to polyhedral formation). A few empty particles lacking cores are arrowed (x 12 000).

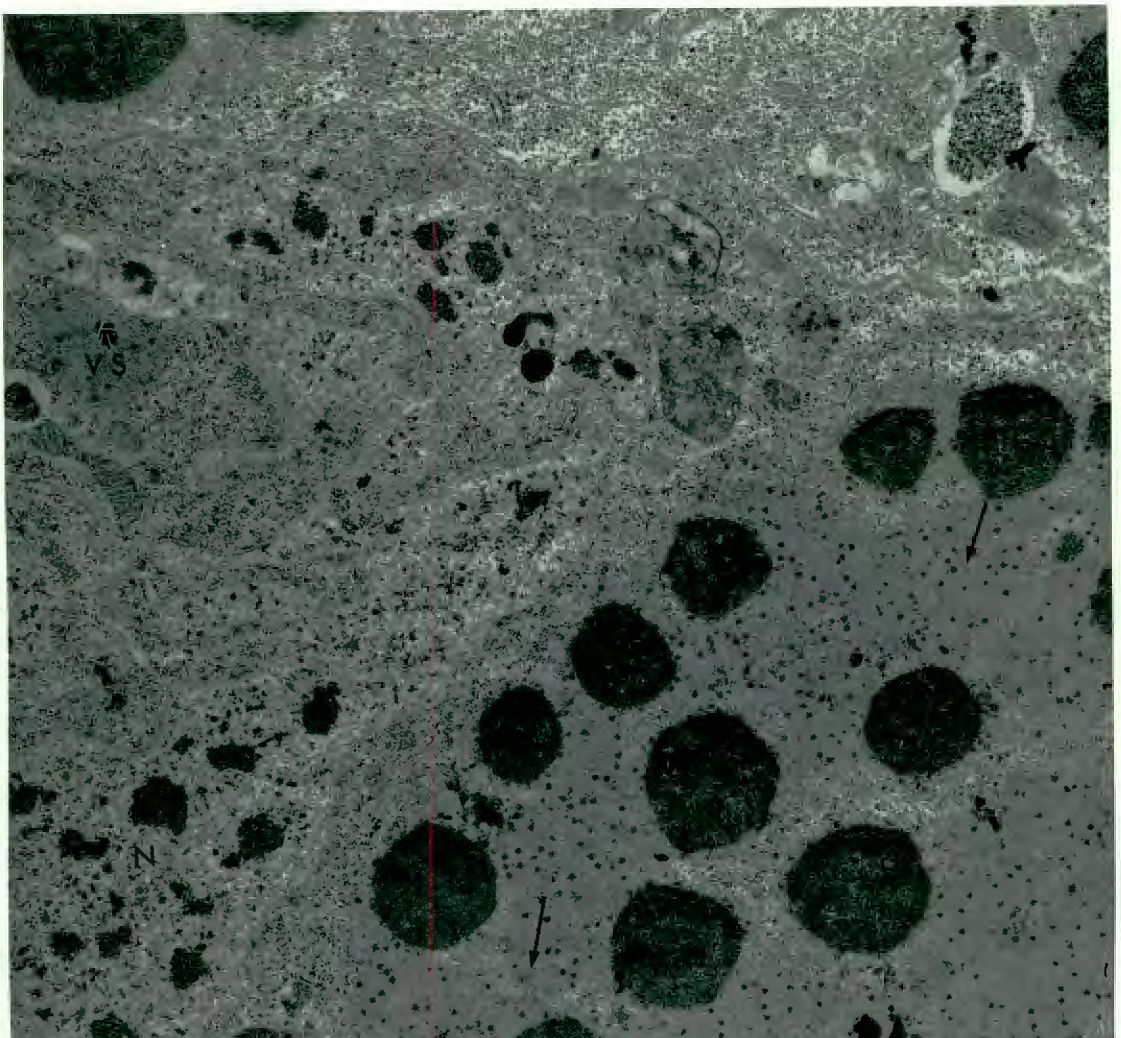
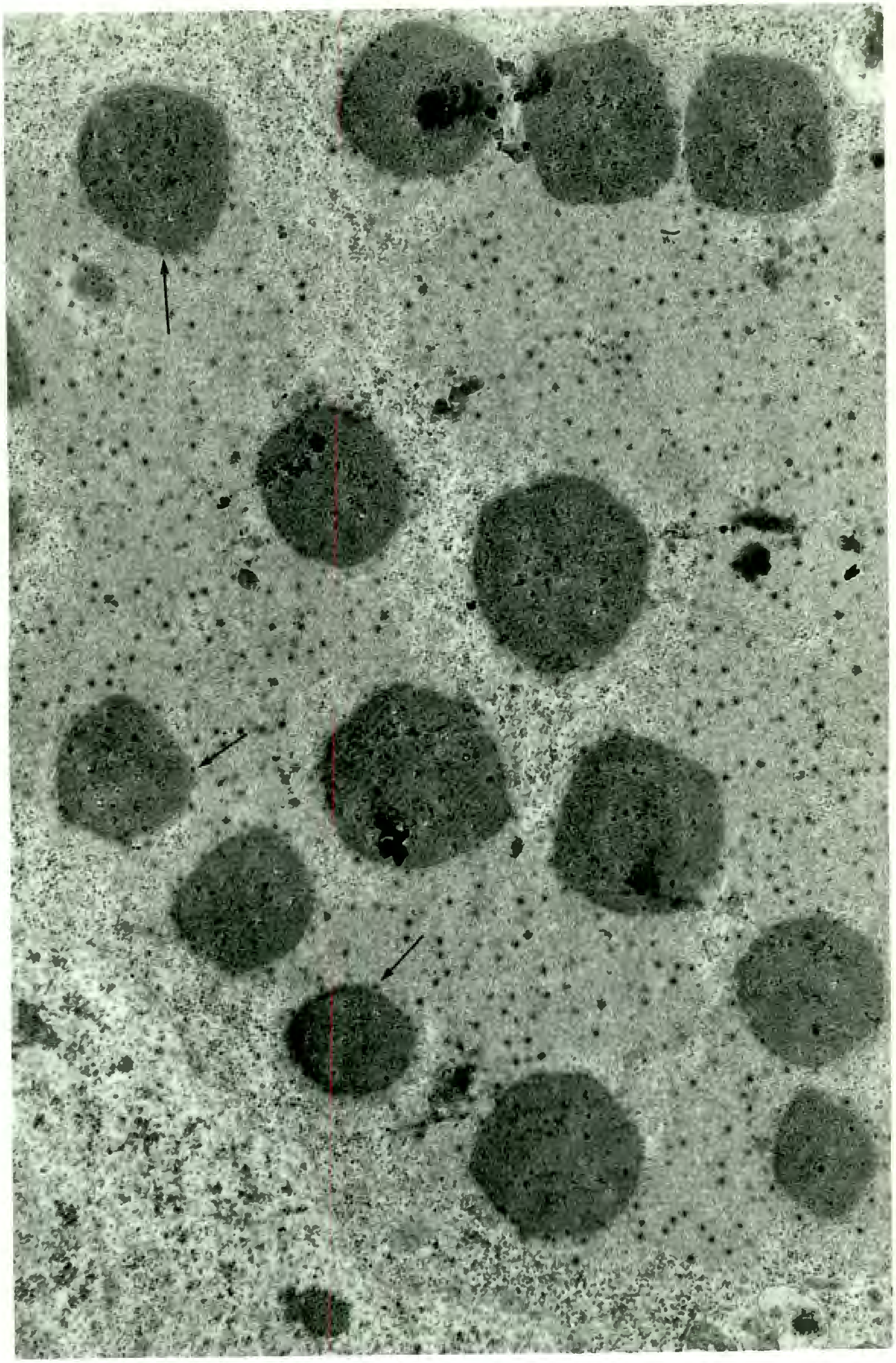

VS
N
CM
P
VP

Fig. 6.17 Thin section of CPV-infected columnar epithelial cell at higher magnification. The crystalline nature of the polyhedra is illustrated and virus particles in the process of incorporation within polyhedra can be recognized (arrowed). Virus particles show a dense core and external capsid. A membrane-like structure can be seen enclosing the large polyhedra, but is not evident in immature polyhedra (x 24 000).



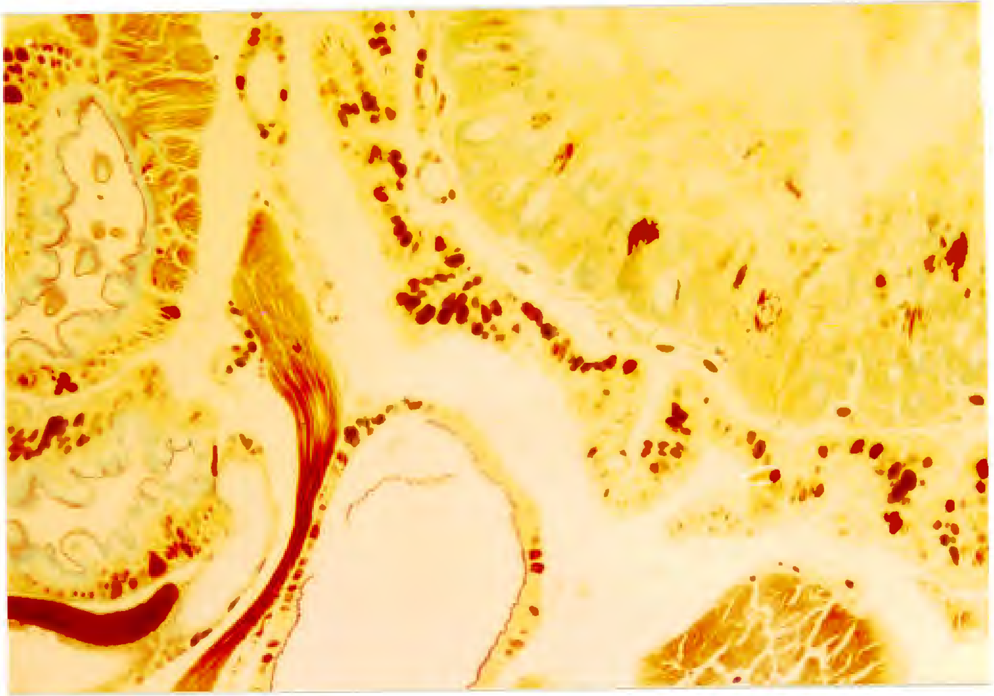


Fig. 6.18 Section through *H. armigera* larva three days after infection with a field mixture of NPV and CPV. Note the variety of tissues that are infected (x 400).

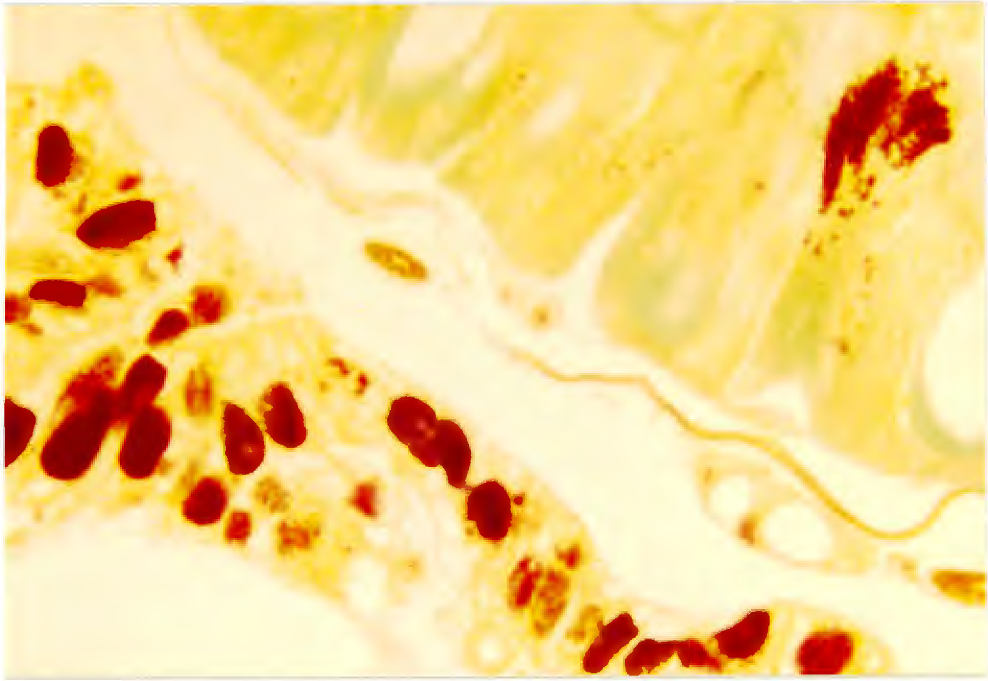


Fig. 6.19 Higher magnification of the above field. Note the scattered CPV polyhedra in the columnar epithelial cell whereas fat body nuclei are filled with NPV polyhedra (x 1500).

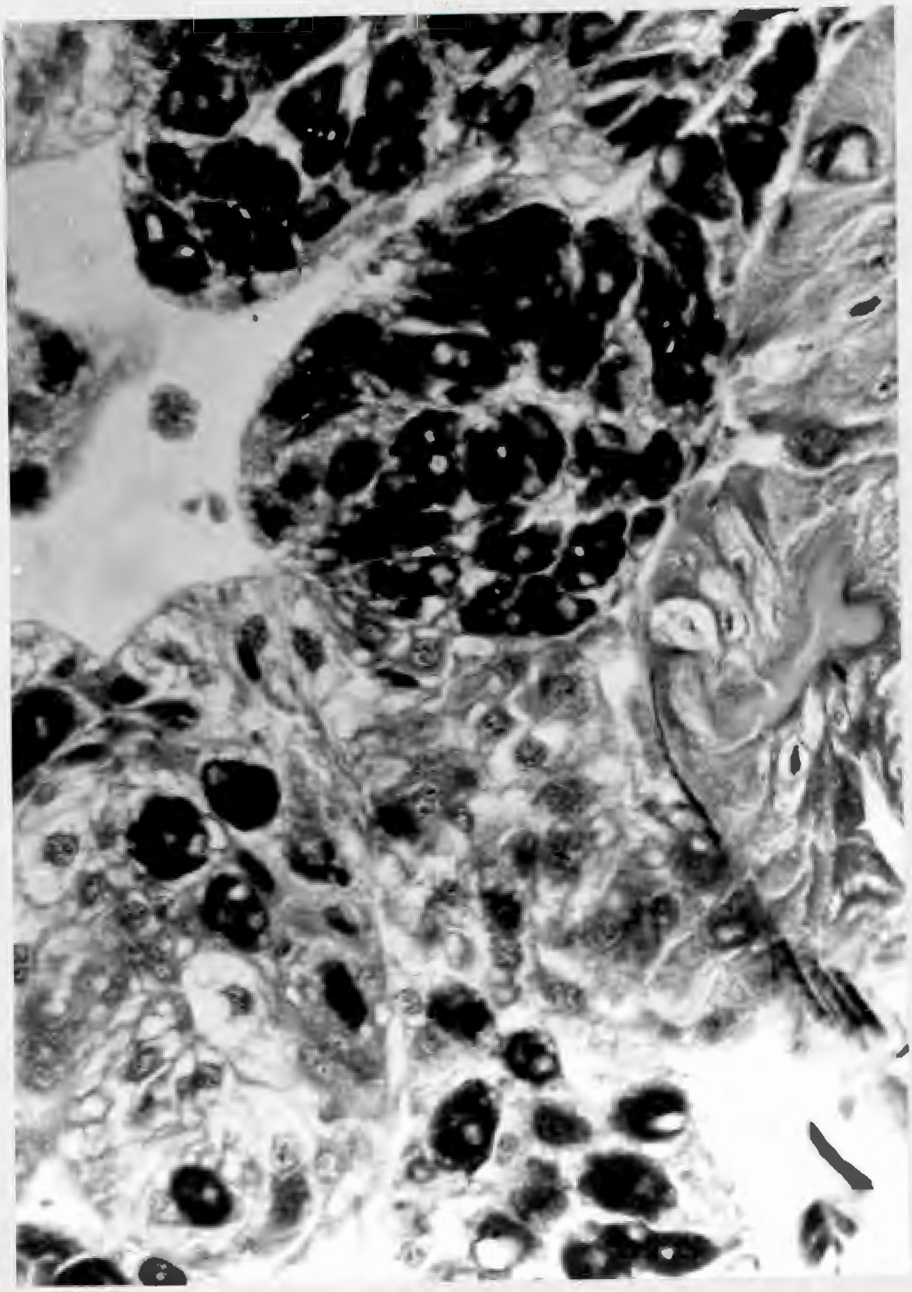


Fig. 6.20 Transverse section of *H. armigera* larva infected with GV. Note the hypertrophied fat body cells infected with GV and densely-staining network in the nuclear area extending to the cytoplasm. Normal-looking fat cells are adjacent to infected cells (x 1200).

Fig. 6.21 Thin section through infected larval fat body cell five days after infection with GV. Note the GV capsules at different stages of development (x 15 000).

A = Longitudinal section of mature GV, i.e. occluded within capsule,

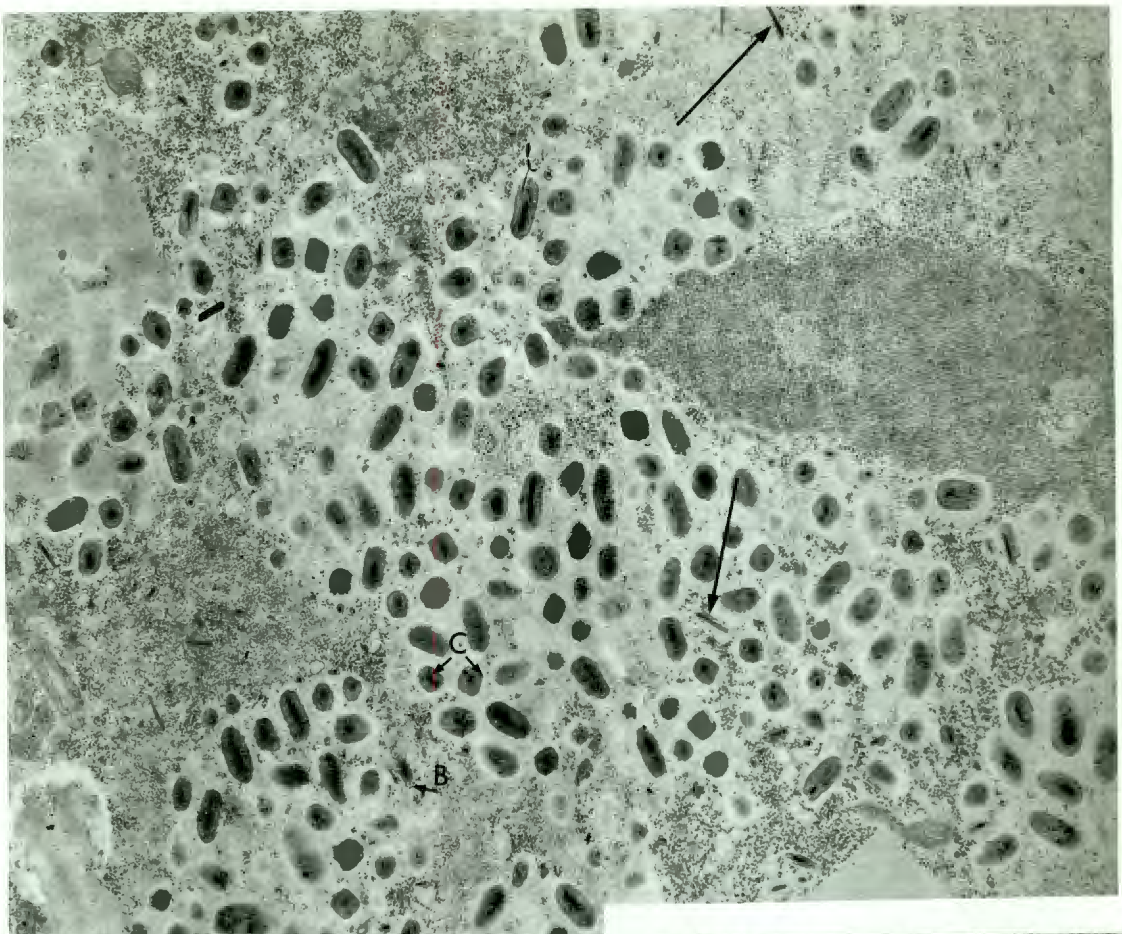
B = Maturing capsule,

C = Transverse section of GV,

→ = Viral membrane

6.21.1 Note the distinct viral membrane in the unencapsulated particles (x 30 000).

6.21.2 Note the nucleocapsids (n) enveloped by a triple-layered virus membrane (m), and a space between the virus membrane and nucleocapsid filled with material of low electron density. The crystalline lattice in protein capsules can be seen (x 50 000).



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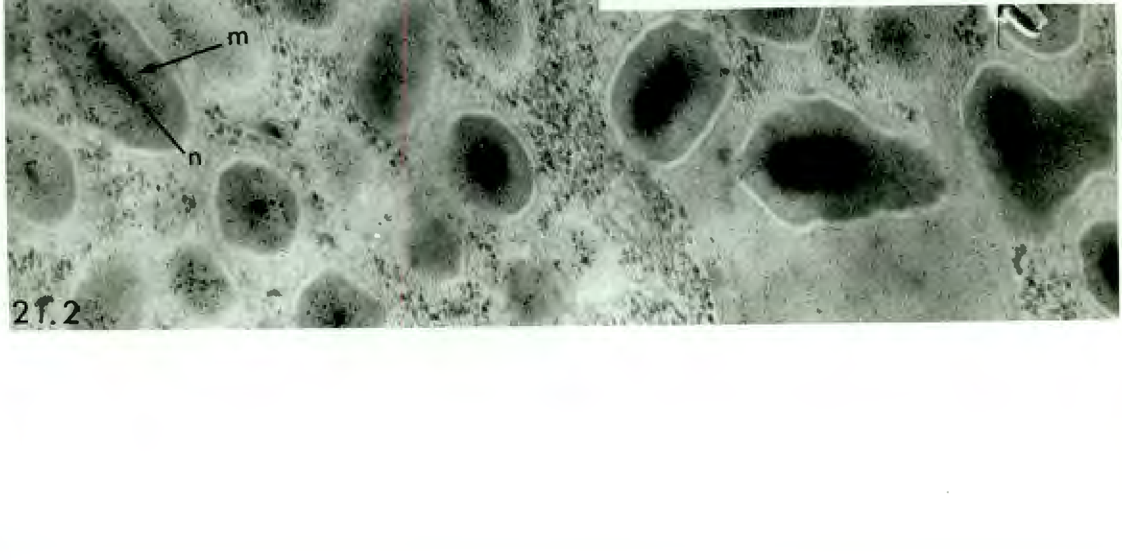
21.1



21.2

were examined in the electron microscope. Many virus particles were evident, either with a prominent capsule or in the process of acquiring capsules (Fig. 6.21). The virus particles appeared to be grouped in areas of the nucleoplasm that were clear of chromatin. By day 5 the nuclear membranes were no longer visible, suggesting that the GV particles had extended into the cytoplasm, where they lay between the mitochondria and other organelles of the disintegrated cytoplasm.

The multiplicity of particle shapes is explained at least in part by the angle of section. Generally they were seen to be rod-shaped virions surrounded by a less dense capsule (crystalline in appearance) giving rise to long and short ovoid bodies, but many in transverse or tangential section were spherical or quadrangular. In these the membrane surrounding the virion was seldom definable, but in the non-enveloped virions the membrane was quite clear in longitudinal or transverse section (Fig. 6.21.1). A space between the nucleocapsid and the virion was seen both in the non-enveloped particles and in the enlargement of the enveloped particles (Fig. 6.21.2).

Examination of some of the fat body cells from mixed infections, revealed both GV and NPV present in the same cell; with GV predominating in a different part of the cell.

6.3 DISCUSSION

Histopathological studies were undertaken to examine which changes at the tissue or cellular level were due to viral infection with CPV, NPV, GV, or a mixture of these viruses. Studies on the infectivity of the different viruses indicated that viruses interacted in mixed infections. It was important to ascertain whether there was interference in mixed infections and which specific tissues were infected by the different viruses. In the experiments described in this chapter, it was possible to demonstrate major changes in the cells and organs of the midgut region of *H. armigera* after the larvae had become infected by feeding on a diet contaminated with a specific virus, either NPV, CPV,or GV.

The method of study was to examine tissue sections for gross cytopathological changes by light microscopy and for finer changes in thin sections by electron microscopy. For the former, the modified Azan staining of Hamm (1966) was used to demonstrate viral replication sites and synthesis. In the case of NPV- and CPV-infected tissue, the bright red within the infected cells pointed specifically to massed polyhedra in the nucleus or cytoplasm. This specific staining was not seen in uninfected larvae or in uninfected cells adjacent to those in which the virus particles were being synthesized and assembled.

In the present investigations, infection by the oral route - the presumed route in natural infections - led to virus replication with a consequent intracellular disorganization that varied for the different viruses. CPV of *H. armigera* was found to infect and replicate almost exclusively in the mucosal columnar epithelial cells. In contrast, the CPV infecting *Hemerocampa pseudotsugata* (Martignoni *et al.*, 1969) was detected in both goblet and columnar epithelial cells. However the positioning of the CPV polyhedra in *H. armigera* cells was similar to that described by Martignoni *et al.* (1969), i.e. they were apicobasally oriented. Polyhedra started to appear 48 h after infection and were clearly visible after 72 h. This is a similar time lapse to that found by Bird and Whalen (1954). Replication took place in the cytoplasm, which showed early degeneration of the intra-cellular organelles.

Despite this, the epithelial cells maintained their staining properties and structural integrity for as long as nine days after infection.

CPV-infected larvae, not killed for study, reached maturity with no sign of disease despite the large numbers of polyhedra present (Fig. 6.10). This finding was similar to that of Stairs *et al.* (1968) in a study on the CPV infection of *Estigmene acrea*. Bird and Whalen (1954) also found that many of the CPV-fed larvae survived, and they suggested that the infected midgut cells slough off and are replaced by new cells.

Striking differences in morphology and in the processes of replication were seen in the three specific infections in thin sections examined under the electron microscope. In cells infected with CPV, the virions in varying numbers were distributed within the spherical angulated crystalline matrix to form a polyhedron. Large numbers of particles developed within a bed of finely granular viroplasm in the cytoplasm of the cells, with resultant degeneration or pushing aside of the structures inside the cell. There were more virions free in the cytoplasm than occluded within polyhedra. This finding was similar to observations by Stairs *et al.* (1968) and Hayashi (1970). In the former study, many nonoccluded particles were observed in the cytoplasm; these were complete particles, with well developed cores similar to those occluded within polyhedra. The occluded particles were more compact and spherical than the nonoccluded particles. A similar situation was observed in *H. armigera* infected with CPV; the particles within polyhedra appeared smaller because they were presumably compacted by the polyhedral protein. Hayashi (1970) found that more than 70% of the particles were in the free state.

Both the virogenic stroma in which virus particles develop, and the crystallogenic matrix which surrounds developing polyhedra (Arnott *et al.*, 1968), were seen in sections of CPV-infected *H. armigera* cells. In the study by Arnott *et al.* (1968), incomplete particles predominated in the virogenic stroma, while the free particles present in the crystallogenic matrix were complete. Only complete particles, i.e. particles containing nucleic acid, were observed in both structures in the present study. These free particles are probably as infectious as occluded particles and may be the means of infecting healthy cells.

Morris (1966), who studied histochemical changes in *Melolontha melolontha*, suggested a hypothesis regarding the mechanism of CPV proliferation. He found a decrease in nuclear RNA accompanied by an increase in cytoplasmic RNA; the finding suggested movement of nucleolar RNA into the cytoplasm. Since the CPVs have been shown to be ds RNA, this appears likely.

In the thin sections of CPV-infected *H. armigera* midgut cells, polyhedra and virus particles were seen only in the cytoplasm, as is typical of most CPVs (Smith, 1967). This is in contrast with the observation made by Hukuhara and Yamaguchi (1973) that a CPV strain of *B. mori* produced inclusions almost solely in the nucleus.

In animals feeding on plants the electrical potential difference across the cell membrane is due to the high internal and low external concentration of K ions. Plant material contains high concentrations of K ions, and the excess cations must be removed. This is usually performed by malpighian tubules but in the case of *Cecropia*, Anderson and Harvey (1966), in their work on midgut fine structure, showed that the midgut and probably the goblet cells are involved in this process.

The close proximity of mitochondria and cell membrane (in the microvilli) is usual in cells actively involved in transport. This hypothesis regarding the roles of goblet cells is of interest with regard to CPV infection of *H. armigera*. The presence of CPV polyhedra in columnar epithelial cells and not in goblet cells allows the normal functioning of these cells to continue throughout infection. This may explain why there is no apparent adverse effect on the insect. In addition, the disease does not cause flaccidity of the insect, and the active transport of ions continues via the uninfected malpighian tubules and possibly the goblet cells. On the other hand, NPV-infected *H. armigera* show marked flaccidity possibly because of the non-functioning of infected malpighian tubules and goblet cells.

Tissues infected by NPV include a variety of submucosal cells and tissues of the fat body, the tracheoles, the silk glands, the malpighian tubules, the hypodermis and the sarcolemma of muscle cells. Similar observations were made by Livingstone and Yearian (1972), Mathad *et al.* (1968), Adams and Wilcox (1968), and Vail *et al.* (1972). Observations made in the present study show that NPV in *H. armigera* was synthesized and assembled in the nucleus. Following hypertrophy of the nucleus, disruption of the polyhedra-laden cells resulted in release of polyhedra. The mucosal epithelium was not markedly affected by NPV but appeared shrunken, and it is possible that its function was impaired.

It has been suggested (Bergold, 1963b) that the number of nucleocapsids within viral envelopes may be host specific. The maximum number in fat body cells of *P. dispar* was 21 (Harrap, 1972c), whereas in *Cadra cautella* the number ranged from 2 to 23 nucleocapsids and in *Plodia interpunctella*, from 3 to 33 (Hunter *et al.*, 1973b). Kawamoto and Asayama (1975) undertook an electron microscopic study on the relationship between the number of nucleocapsids and their arrangement pattern within envelopes in NPV-infected larvae of *Euproctes similis*. They found that the number of nucleocapsids ranged from 1 to 39, and that the central row was always composed of the largest number of nucleocapsids, with successive rows on either side consisting of progressively smaller numbers of nucleocapsids to form a polygonal shape. The assembly of NPV of *H. armigera* was characterized by the production of prominent rod-shaped nucleocapsids, arranged in close contact and in an apparent orientation that led to varying patterns similar to those described by Kawamoto and Asayama (1975). The longitudinal, tangential, and cross-sectional views of the nucleocapsids (Fig. 6.7) suggested an irregular packing arrangement of the type (5-6-7-8-7-6) with a total of 39 nucleocapsids. A different packing arrangement (1-2-3-4-5-4-3), with a total of 22 nucleocapsids, was also seen, indicating that different arrangements of nucleocapsids could be present within the same host. This was seen by Adams and Wilcox (1968), who observed viral particles both singly and in packets.

The histological changes observed in *H. armigera* infected larvae were sufficiently different for each virus to allow it to be recognized readily in sections of insects doubly-infected with CPV and NPV. It was found that CPV replicated only in the midgut whereas NPV infected a variety of tissues. These observations are similar to those made by Bird and Whalen (1954) on NPV- and CPV-infected *Choristoneura fumiferana* and those of Harpaz *et al.* (1965) on CPV- and NPV-infected *Thaumetopoea wilkinsini*.

It has been shown in the present study that the major site of GV infection is the fat body cells. The replication of GV in the nucleus of infected cells was readily recognized because of the unique morphology of the enveloped particle. The nucleocapsids were rod-like bodies with prominent 'capsules' and were surrounded by a clear space and a membrane that was clearly visible in some of the enveloped virions and non-enveloped particles (Fig. 6.21.1). Tanada and Leutenegger (1970) found GV nucleocapsids and complete particles but no encapsulated particles in larval midgut cells of *Trichoplusia ni*. In the present study, examination of histological sections of GV-infected larvae revealed no infection of midgut cells.

The close similarity of two GVs and two NPVs was established by Beaton and Filshie (1976), who showed in optical diffraction studies that the two viruses had an identical lattice structure. In the present study, the nucleocapsid rods of both NPV and GV of *H. armigera* looked very similar. However, the nucleocapsids of NPV were packed together whereas the GV capsule contained only one nucleocapsid. This allowed easy identification of the two virus types in fat body cells infected by both viruses.

Since only CPV and not NPV was found to infect the midgut cells, infected midguts could be used as the source of CPV for further physico-chemical studies. The CPV was isolated free from contaminating virus. No evidence of NPV replication was found in midgut cells in mixed infections of *H. armigera*. In mixed infections of NPV and CPV, the quantity of NPV was much greater than that of CPV. The NPV increased more rapidly than CPV and was widely distributed in the tissues. By the time larvae died from NPV in mixed infections, relatively little CPV had been produced. This may account for the difficulties experienced in separating the two viruses early in this study.

CHAPTER 7

BIOPHYSICAL PROPERTIES OF THE CPV AND NPV VIRUS PARTICLES

7.1 BUOYANT DENSITIES OF *Heliothis armigera* CPV AND NPV

The principle of isopycnic sedimentation was introduced by Linderstrøm-Lang (1937). In this technique, the density gradient column encompasses the entire range of densities of sample particles. The particles sediment to positions in the gradient where the gradient and particle density are equal. The isopycnic technique separates particles entirely on their buoyant density differences. The supporting concentration gradient may be preformed or formed by redistribution of the gradient material during centrifugation (Meselson *et al.*, 1957). CsCl is frequently employed in isopycnic centrifugation to reach the high densities required with some materials (Kunkel *et al.*, 1959).

Isopycnic centrifugation is valuable both for purification of viruses and for the determination of their buoyant densities.

7.1.1 Method

Stock solutions of CsCl were prepared by dissolving the anhydrous salt (BDH Analar grade) in Tris-HCl buffer, pH 8.0. A range of dilutions with differing densities were prepared from the stock solutions and were layered sequentially in centrifuge tubes. A gradient ranging from 32-47% (*m/m*) consisting of five steps was used for CPV and one ranging from 14-40% (*m/m*) consisting of seven steps was used for NPV. The tubes were left at room temperature for 2 h to allow diffusion to produce a uniform gradient. The purified virus was layered on top and the gradients were immediately centrifuged at 40 000*g* in the SW 50.1 rotor at 20°C for 2 h and 2½ h for NPV and CPV respectively.

After centrifugation, the ISCO fractionater and VA-2 UV analyser were used to collect fractions of 0.2 ml and to monitor the position of the virus band (see Section 3.3.2). For the density measurement, the refractive index of aliquots of each fraction was measured with an Abbé refractometer at 20-22°C. The densities of CsCl standards were determined by pycnometry to confirm values obtained using the formula of Vinograd and Hearst (1962).

The densities of the samples from the gradient were determined both from a standard curve and by using the following formula:

$$\rho^{25} = a\, n_d^{25} - b \qquad \text{(Vinograd and Hearst, 1962)} \; ..... \; (1)$$

where a = 10.8601 and

b = 13.4974 for CsCl for a density range of 1.25-1.90

ρ^{25} = density at 25°C

n_d^{25} = refractive index of the solution at 25°C

7.1.2 Results

After centrifugation to equilibrium the tubes were viewed in the light of a mercury vapour lamp. A narrow band was visible in the CPV gradient and a broader band in the NPV gradient. A typical ultraviolet absorption recording obtained after passing the gradient through the flow cell of the ISCO fractionator is shown together with the density curve for CPV in Fig. 7.1. The mean values and S.D. for the different

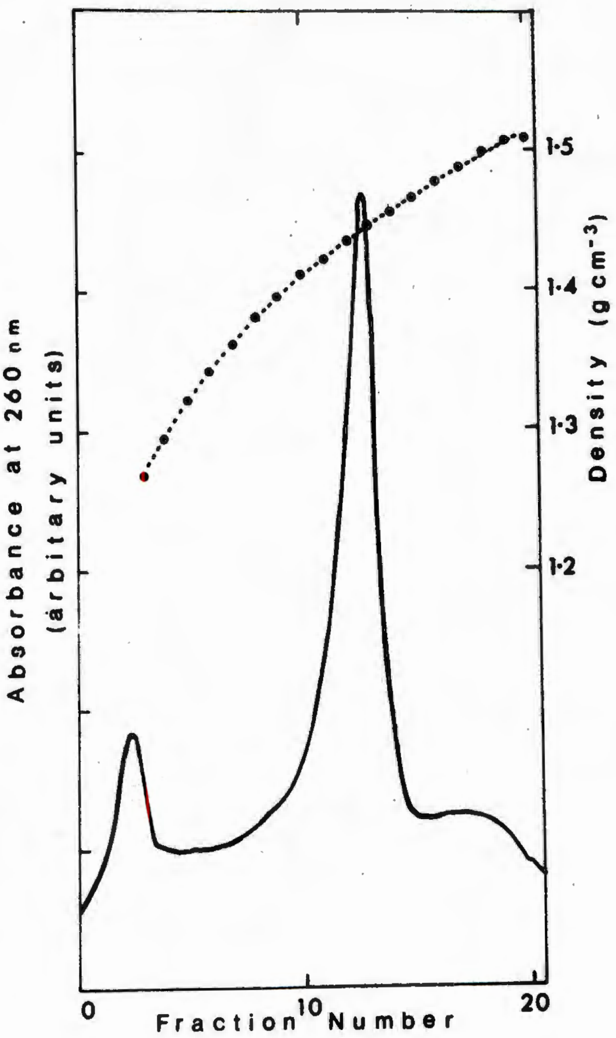


Fig. 7.1 CsCl gradient centrifugation of CPV_{A2} of *H. armigera*

— absorbance at 260 nm

●--● density in grams per cubic centimetres

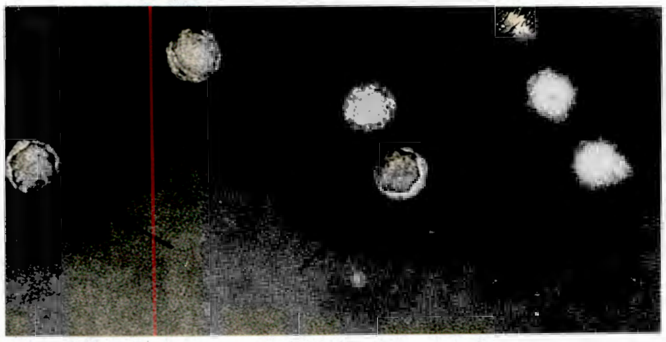


Fig. 7.2 CPV of *H. armigera* from the UV-absorbing peak of the CsCl gradient. Several CPV particles (arrowed) show surface damage (x 120 000).

densities were:

1.444 ± 0.006 g cm^{-3} for CPV$_{A2}$ (free virions), 5 determinations,

1.456 ± 0.001 g cm^{-3} for CPV$_{A2}$ (occluded virions), 2 determinations,

1.241 ± 0.001 g cm^{-3} for NPV, 2 determinations.

The buoyant densities of CPV virions, whether free or obtained by digestion of polyhedra, were similar. It is notable how close the buoyant density determined for CPV in CsCl solution is to the reciprocal of the partial specific volume determined in phosphate buffer ($\bar{v} = 0.69$, $1/\bar{v} = 1.449$). The presence of virus in UV-absorbing fractions of the gradient was confirmed by electron microscopy (Fig. 7.2). The electron micrographs revealed that some *H. armigera* CPV particles showed surface damage after exposure to CsCl.

7.2 DETERMINATION OF THE SEDIMENTATION COEFFICIENT

The sedimentation coefficient (s) of a substance depends on its mass, shape, and density, and is estimated by the rate of displacement of material down a cell in a centrifugal field. It is the velocity per unit field, measured in Svedberg units (S) ($1S = 10^{-13}$ s; Svedberg and Pedersen, 1940). Knowledge of s is necessary to establish optimum conditions for centrifugation while isolating viruses.

The sedimentation coefficient can be defined as follows:

$$s = \frac{dr/dt}{r\omega^2} = \frac{d\ln r}{\omega^2\, dt} \qquad \text{..... (2)}$$

where s = sedimentation coefficient

r = distance from the axis of rotation (cm)

ω = angular velocity (radians/s)

t = time (s).

A plot of log r against t should yield a straight line from which the slope can be measured and s calculated. Sedimentation coefficients are conventionally corrected to standard conditions at 20°C with pure water as solvent using the formula:

$$s_{20.w} = s_{obs} \frac{\eta_{obs}(1-\bar{v}\rho)_{20.w}}{\eta_{20.w}(1-\bar{v}\rho)_{obs}} \quad \ldots\ldots (3)$$

where s_{obs} = sedimentation coefficient observed

$s_{20.w}$ = sedimentation coefficient when pure water at 20°C is the solvent

$\bar{v}$ = partial specific volume

ρ = density of solution

η = coefficient of viscosity

Since s varies with concentration of solute, sedimentation rates are conventionally reported as s_o after extrapolation to zero concentration.

7.2.1 Method

The samples studied were: (i) virions from *H. armigera*, a partially purified mixture of NPV and CPV_{A1}; (ii) purified CPV_{A2} of *H. armigera* alone; (iii) a mixture of the CPV and ε particles from *N. cytherea cytherea* previously purified by zone electrophoresis. Samples were each concentrated by centrifugation and the pellets resuspended in 0.8 ml of phosphate buffer, pH 7.0 (PB).

Sedimentation velocity measurements were made at 20°C at speeds from 6166 to 14 000 rpm in a Beckman Spinco Model E Analytical

Ultracentrifuge using the standard methods described by the manufacturer (Chervenka, 1969). Schlieren optics, and exposure intervals of 4, 8, or 16 min and phase angles of 40^0 or 50^0 were used. The position of the boundary on each photograph was measured by means of the Nikon Profile Projector (Model 6CT2).

The distance r from the centre of rotation to the boundary was calculated for each photograph and log r was plotted against time to obtain the slope.

7.2.2 Results

Sedimentation Coefficient Determination

A series of sedimentation velocity experiments was performed. Figures 7.3 and 7.4 are Schlieren photographs of the sedimentation of:

(i) a mixed preparation, i.e. NPV and CPV separating into two distinct peaks, and

(ii) pure CPV sedimenting as a single component.

The sedimentation coefficients calculated using equations (2) and (3) for CPV and NPV of *H. armigera* and the CPV and ε particles of *N. cytherea cytherea* are listed in Table 7.2.

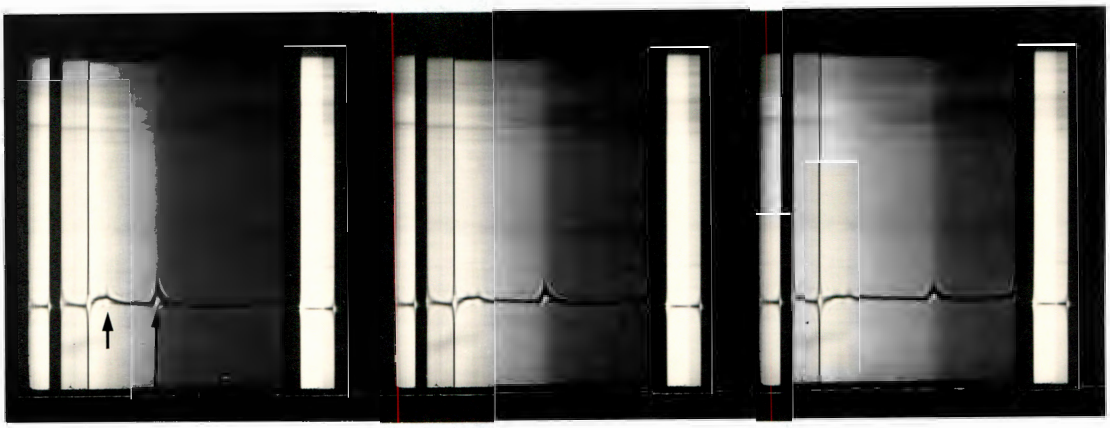


Fig. 7.3 Analytical sedimentation pattern of CPV (↑) and NPV (↑) virus particles. Sedimentation is from left to right. Rotor velocity was 8766 rpm at a temperature of 20°C, and exposure intervals of 4 min. The Schlieren angle was 60° and the medium was 0.14 M NaCl, pH 7.0.

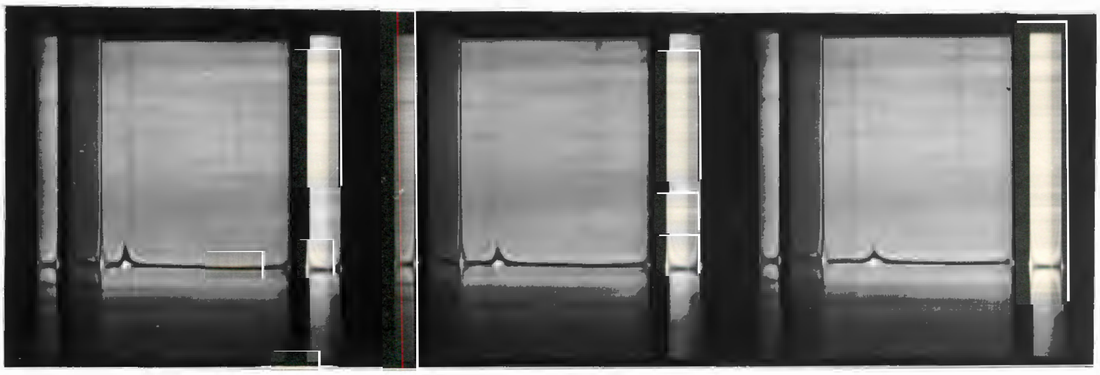


Fig. 7.4 Analytical sedimentation pattern of pure CPV particles. Sedimentation is from left to right. Rotor velocity was 8766 rpm at a temperature of 20°C and exposure interval was 8 min. The Schlieren angle was 50° and the medium was 0.14 M NaCl, pH 7.0.

TABLE 7.2

SEDIMENTATION COEFFICIENT DETERMINATIONS

Host	Virus	Speed (rpm)	Exposure intervals (min)	Calculated $s_{20.w} \times 10^{-13}$ s
H. armigera	NPV	8 766	4	1 188
	CPV_{A1}	8 766	4	374
	CPV_{A2} free virus	8 766	8	389)
	CPV_{A2} "	8 000	4	407)
	CPV_{A2} "	6 166	16	386)
	CPV_{A2} "	8 766	8	408)
	CPV_{A2} "	12 590	8	407)
	CPV_{A2} free virions (a)*	9 341	8	384
	CPV_{A2} occluded virions (b)†	9 341	8	384
N. cytherea cytherea	CPV particle	14 290	8	418
	ε particle	14 290	8	200

Mean value 399 ± 10.92

H. armigera CPV_{A1} = CPV from field isolates.

CPV_{A2} = CPV from laboratory-reared larvae.

When a sample of CPV free virions (a)* and occluded virions (b)†, i.e. particles liberated from polyhedra of *H. armigera* were centrifuged in the same rotor using a wedge window, no difference was found in the calculated sedimentation coefficients.

7.3 DETERMINATION OF PARTIAL SPECIFIC VOLUME

A knowledge of the precise partial specific volume ($\bar{v}$) of a virus is necessary for molecular weight (mol. wt.) determinations by sedimentation. This quantity occurs in the term $M(1-\bar{v}\rho)$ and an uncertainty of 1% in $\bar{v}$ will cause an uncertainty of about 3% in the mol. wt. calculations. The partial specific volume is defined as the volume increase (ml) when 1 g of the solute is added to an infinite volume of the solution and is equivalent to the reciprocal of the effective density of the solute when it is in solution. It is determined either by:

(i) calculation from amino acid composition;

(ii) densitometry; or

(iii) sedimentation studies in solvents differing in density.

The first is most popular because it involves least work, but it is only approximate, even when applied to purified proteins. The second method requires large quantities of material and is a tedious procedure, the accuracy of which is severely limited by difficulties in determining concentrations. The last method does not require knowledge of protein concentrations, and small amounts of material are adequate.

Partial specific volumes in this study were determined by sedimentation in solutions of various H_2O/D_2O ratios according to the technique of Bellamy *et al.* (1974). To allow for deuterium exchange, the mol. wt. is increased by a factor k, where k is the ratio of the mol. wt. of the protein in D_2O/H_2O mixture to that in pure water. The value k can be estimated from the number of exchangeable hydrogen atoms in the solute molecules (Hvidt and Nielsen, 1966). A value of $k = 1.0155$ was obtained by direct measurements of the increase in weight of bovine plasma albumin and other proteins on deuteration and should be constant for all proteins (Martin *et al.*, 1959; Hvidt and Nielsen, 1966).

7.3.1 Method

The method of Bellamy *et al.* (1974) was modified and used to measure $\bar{\nu}$. Two single-sector cells (12 mm) were used; one cell was half-filled with one solution under investigation and the other was completely filled with the second solution. (When material was scarce, both cells were half-filled and fluorocarbon was added to one cell.) The top half window of the first cell and the bottom half of the second cell were blacked out with a felt-tip pen (Fig. 7.5). This allowed two adjacent images to be obtained on the photographic film. In this simple way it was possible to obviate the need for an expensive ultraviolet optics multiplexer.

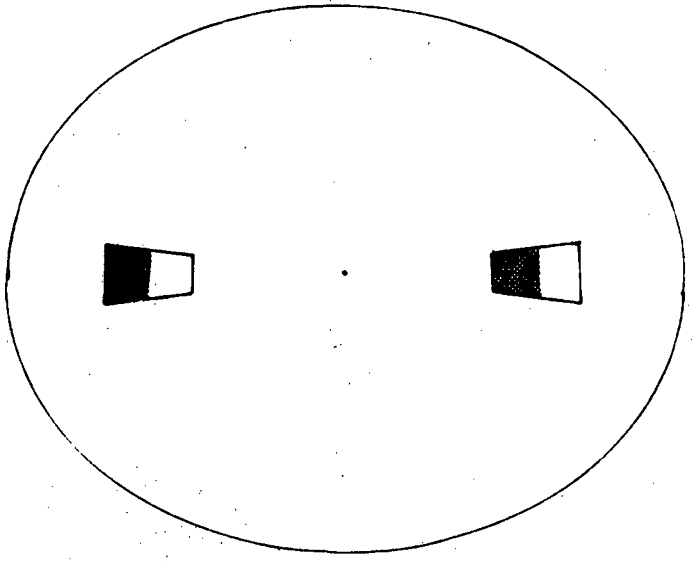


Fig. 7.5 Two single-sector cells in the same rotor. The top half of one and bottom half of the other were blacked out so as to obtain two adjacent images.

The buffer was 0.2 M KCl, 0.06 M PB, ionic strength 0.33. D_2O (99.75%, Uvasol for spectroscopy) was obtained from Merck. CPV free virions were extracted from 200 larvae of *H. armigera* and purified as described in Section 3.3.

The purified virus was exhaustively dialysed against the buffer for three days at 4°C. Samples were prepared with different proportions of D_2O (Table 7.3) and equilibrated overnight at 4°C before use.

TABLE 7.3

PROPORTIONS OF H_2O AND D_2O FOR $\bar{v}$ DETERMINATION OF CPV

Virus (ml)	H_2O (ml)	D_2O (ml)	D_2O %
0.4	1.2	0	0
0.4	0	1.2	75

The prepared samples were placed in the cells in an AN-J rotor, and then equilibrated in the ultracentrifuge for 30 min with the temperature control set at 20°C; the temperature was controlled at the end of the experiment. A suitable speed was 12 000 rpm. Ultraviolet optics at a wavelength of 260 nm were used with the slit wide open and photographs were taken with Ilford N5.31 film. Buffer densities were determined by pycnometry.

7.3.2 Results

The partial specific volume of CPV_{A2} of *H. armigera* was determined by sedimentation in D_2O and H_2O. A typical sedimentation figure for CPV in the two solvents obtained with ultraviolet optics is shown in Fig. 7.6. The use of two cells, shaded in opposite regions, gave clear images with no overlap. A plot, typical of Δ log r versus t, is depicted in Fig. 7.7. Linear regression was used to obtain the slope of this plot for virus sedimenting in H_2O and D_2O. The magnification factor was calculated by comparing dimensions of the cell and the photographs. Sedimentation coefficients in H_2O and D_2O were calculated using equation (2). Both the sedimentation coefficients and their ratios in D_2O and H_2O are listed in Table 7.4.

TABLE 7.4

SEDIMENTATION COEFFICIENTS DETERMINED IN SOLUTIONS OF D_2O/H_2O

$s_{20.w}$			
Run	D_2O	D_2O	Ratio
	0%	76.68% (by weight)	
1	416.4	314.6	1.3236
2	497.7	290.0	1.3676

Fig. 7.6 Sedimentation of CPV$_{A2}$ of *H. armigera* in the analytical centrifuge in heavy water (A) and water (B). The speed was 12 000 rpm and temperature 20^{0}C. Ultraviolet optics were used at 260 nm. The exposure intervals were 2 min for 8 exposures followed by an 8 min interval. Sedimentation is from left to right. 0.06 M PB, 0.2 M KCl, pH 7.0 I = 0.33 was used.

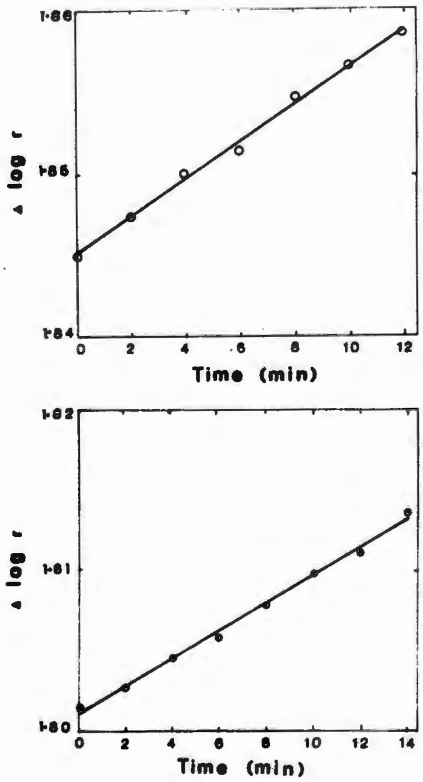


Fig. 7.7 Sedimentation velocity of CPV$_{A2}$ of *H. armigera*. A typical plot of Δ log r versus time for sedimentation in:

water ○
heavy water ●

(Centrifugation was as in Fig. 7.6)

Since $s \propto \frac{M}{\eta}(1-\bar{\nu}\rho)$,

$$\frac{s_1}{s_2} = \frac{1}{k}\frac{\eta_2}{\eta_1}\frac{(1-\bar{\nu}\rho_1)}{(1-\bar{\nu}\rho_2)}$$

$$s_1k\eta_1\ (1-\bar{\nu}\rho_2) = s_2\eta_2\ (1-\bar{\nu}\rho_1)$$

$$\bar{\nu} = \frac{s_2\eta_2 - s_1k\eta_1}{s_2\eta_2\rho_1 - s_1k\eta_1\rho_2} \qquad \ldots\ldots (4)$$

where s_1, s_2 = sedimentation coefficients in H_2O and 76.68% D_2O respectively;

ρ_1, ρ_2 = the respective densities; and

η_1, η_2 = respective viscosities.

The factor k which allows for the exchange of hydrogen atoms is relatively constant for all proteins. A value of 1.0155 for k has been determined for a number of proteins in studies of deuterium exchange in pure D_2O. From a value of 1.015 for 99.9% D_2O, and the approximate composition of the virus (about 74% protein) k was calculated to be 1.0139.

Viscosity figures for D_2O/H_2O mixtures were taken from Klotz and Keresztes-Nagy (1963). From a graph of the viscosity of D_2O versus the percentage of D_2O, a value of 1.1243 was obtained for the viscosity ratio of 76.68 and 0% D_2O.

The density of H_2O = 0.99823 and the density of D_2O,76.68% (by weight) was calculated to be 1.07820. (International Critical Tables.)

The data given above were inserted in formula (4). A mean value for the partial specific volume of CPV of *H. armigera* from two experiments was calculated to be:

$$\bar{\nu} = 0.69 \pm 0.02 \text{ ml g}^{-1}.$$

7.4 DIFFUSION COEFFICIENT DETERMINATION

The diffusion coefficient depends on the shape, molecular size, and charge of the particle.

The theory of diffusion is based on the fact that the rate of transfer of diffusing material across a very small element of area is proportional to the concentration gradient of the material in the direction at right angles to the area. The diffusion coefficient (D) was first defined by Fick (1855):

$$D = \frac{dm}{dt} \div A.\frac{dc}{dx}$$

where dm = mass diffusing across

A = area

$\frac{dc}{dx}$ = gradient of concentration

dt = short time interval

In the present study, an immuno-chemical procedure based on quantitative gel precipitin tests was used (Polson, 1958, 1976). The theory for the derivation of diffusion coefficients from the quantitative gel precipitin technique is based on equations by Mitchison and Spicer (1949) in their work on bacterial growth inhibition by streptomycin diffusion into agar gel. In the Polson technique, serial dilutions of antigens and specific antibody at a suitable concentration diffuse into an agarose column from opposite directions. The equation derived by Polson (1958) permits calculation of the diffusion coefficient of the antigen relative to that of the antibody if the relative position of the precipitin band is known. It is given by the following:

$$Dg = Db \frac{xg^2}{xb^2} \qquad \ldots\ldots (5)$$

where Dg, Db = diffusion coefficients of the antigen and antibody respectively; and

xg, xb = the distance of the precipitin band from the antigen and antibody menisci when the two reactants are present at optimal proportions.

From this equation, the diffusion coefficient (Dg) of the antigen may be calculated if that of the antibody is known. The position of the precipitin band relative to the menisci at optimal proportions is independent of time and is only dependent on the relative diffusion coefficients of antigen and antibody (the exact position of optimal proportions is determined by interpolation). The diffusion coefficient of the antibody (Db) was assumed to be a constant and the value of $4.7 \times 10^{-7}\ \text{cm}^2\ \text{s}^{-1}$ for IgG was used (Polson and Deeks, 1960).

A semi-micro-apparatus specially designed by Polson (1976) for the determination of diffusion coefficients allows the accurate measurement of the precipitin bands relative to the original menisci of reacting components. Use of short columns (4.08 × 1.5 mm) reduces the volumes of reactants required and the time taken to obtain a reading. As the time required for transfer of a given amount of material over a certain distance is proportional to the inverse square of the distance, reduction of the column length from that in the original apparatus by 3.9 times will reduce the time for appearance of the precipitin line by approximately 90%. In the conventional apparatus, 0.3% (*m*/v) agarose was the lowest usable agarose concentration, whereas by reduction of capillary diameter, the lower limit may be reduced to 0.03% agarose. Low concentrations of agarose minimise the impedance to the diffusing particles in the gel matrix.

A value for the diffusion coefficient is necessary to calculate the mol. wt. of the virus particles using the Svedberg equation (6).

7.4.1 Method

The semi-micro-apparatus used for the determination of the diffusion coefficient of CPV of *H. armigera* by quantitative gel precipitation is illustrated in Fig. 7.8. The apparatus consists of five rectangular perspex segments A-E of dimensions 11 × 1.5 × 0.5 cm. Segments A and E are lids, segments B and D contain the antigen and antibody in buffer, respectively, and segment C is the reaction column containing agarose gel only. Holes 1.5 mm in diameter had been drilled through the bars at regular intervals, the depth of the holes in C being 4.08 mm. Perspex reference cones (0.4 mm in length) positioned between the holes in segment C, facilitated the accurate measurement of the precipitin bands.

The segments were thoroughly cleaned and greased with vacuum grease to form an air-tight seal. Excess grease was removed with ether and cotton wool swabs. Segments D and E (view 2) were moved to shut off the lower surface in C and the agarose solution (0.1%, *m*/v in PB) was introduced into each well in C. After the agarose had set, A and B were moved (view 3) so as to shut off the wells in C. The apparatus was inverted, and antiserum at the appropriate dilution (1/200 determined by preliminary testing) was pipetted into the wells in D (view 4) and was sealed by sliding E across (view 5). Finally, serial dilutions of purified antigen were placed in wells in B (view 5), and segment A was moved to seal the wells in B (view 6). All the wells were lined up in apposition (with antiserum in bottom segment D) so that antigen and antibody were simultaneously brought into contact with opposite ends of the agarose column (view 7), and the apparatus was kept at room temperature for the duration of the experiment. When precipitin bands were visible, their widths and positions were recorded daily using a microcomparator.

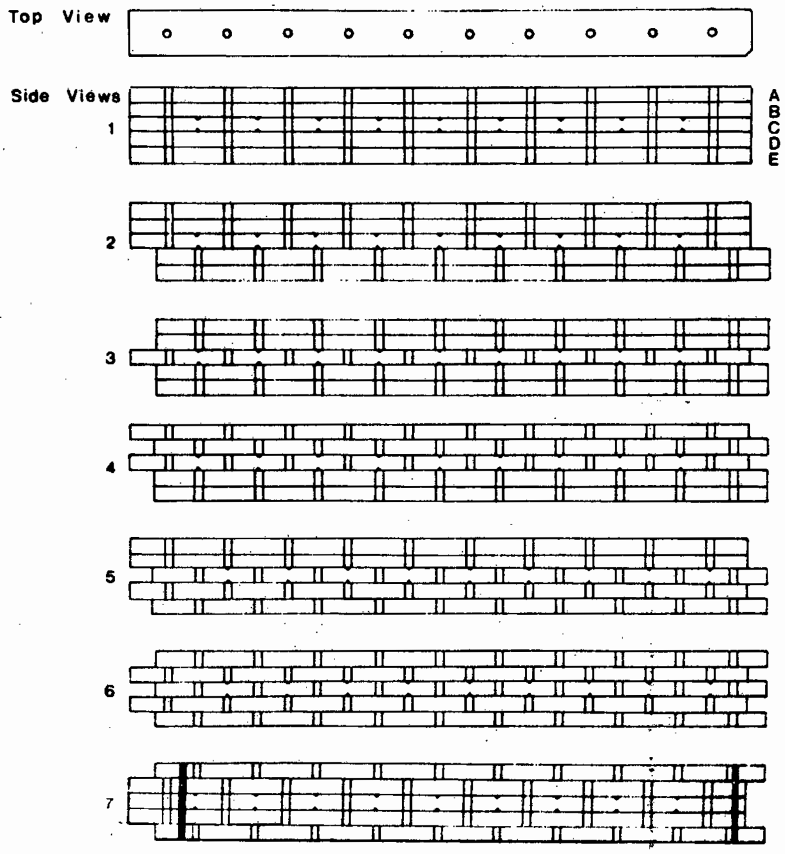


Fig. 7.8 The semi-micro-apparatus used to determine diffusion coefficient using gel precipitation.

7.4.2 Results

Preciptin bands which appeared in wells numbered 1-6 after three days were visible in tubes 4-7 after fourteen days and were still visible in tubes 6 and 7 up to twenty-one days. Measurements of band distances from respective antigen and antibody menisci were made. A photograph of the bands is shown in Fig. 7.9, and a plot of band width versus antigen dilution in Fig. 7.10.

In the zones of antigen excess the bands were diffuse and their low intensity made measurements difficult. In the zones of antibody excess the bands were less diffuse so their positions and widths could be measured. The band in tube 6 did not broaden appreciably with time; this position is close to optimal proportions. A value was read from the linear graph which relates antigen concentration to band position (Fig. 7.10). From the value at antigen dilution of 1/32, the distance of the precipitin band from the antigen and antibody menisci was calculated, and the values obtained were inserted into equation (5).

The diffusion coefficient, D of CPV_{A2} of *H. armigera* was calculated to be:

0.73×10^{-7} cm s^{-1}

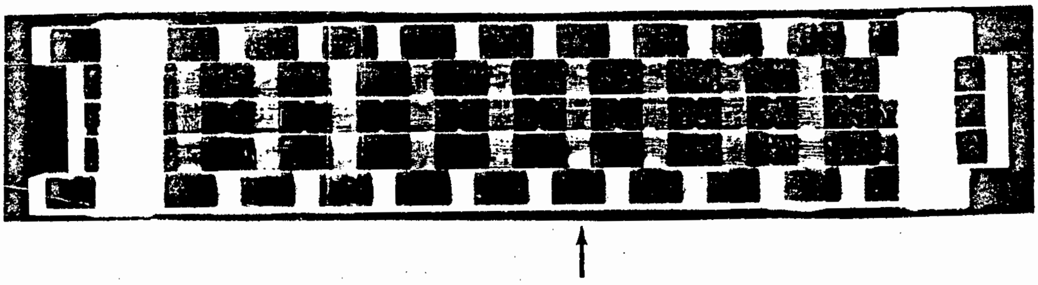


Fig. 7.9 Quantitative gel diffusion of the CPV_{A2} of *H. armigera* with homologous antiserum in 0.1% agarose. Precipitin bands show optimal proportions to be in well 6 (↑). Serial doubling dilutions of virus in the upper wells and a constant concentration of antibody in the lower wells. The precipitin bands are clearly visible only in wells 6 and 7 because scratches on the perspex apparatus were magnified on the prints.

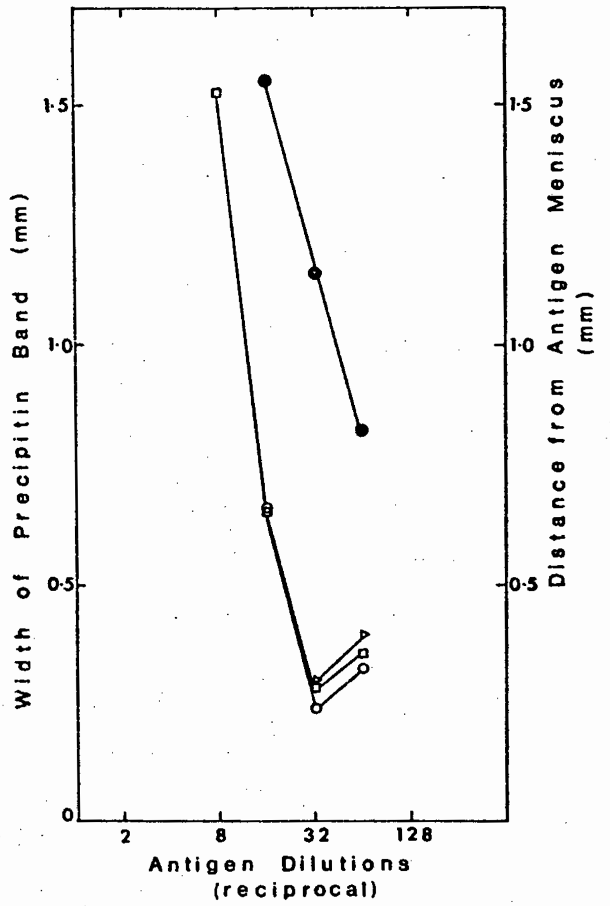


Fig. 7.10 The variation in the precipitin band width in relation to antigen concentration using quantitative gel diffusion.

□—□ 14 days from the start of the experiment
○—○ 18 " " " " " " "
△—△ 21 " " " " " " "

The relationship between the positions of the zones of precipitation and antigen dilutions is also shown. ●—●

7.5 MOLECULAR WEIGHT OF *H. armigera* CPV_{A2} PARTICLES

The molecular weight of viruses is of fundamental importance and of value in various physico-chemical and biochemical studies. Three methods were used in this study to determine molecular weight.

7.5.1 Sedimentation and Diffusion Coefficients

Combination of a sedimentation coefficient (s) measured in the ultra-centrifuge with a diffusion coefficient (D) derived by immunodiffusion. This method relates mol. wt. to s, D and $\bar{\nu}$ (Svedberg and Pedersen, 1940).

$$M = \frac{RTs_{20.w}}{D_{20.w}(1-\bar{\nu}\rho)} \qquad \ldots\ldots (6)$$

R = universal gas constant

T = absolute temperature

$\bar{\nu}$ and ρ have been defined.

Several parameters are required for calculation of the molecular weight. These have been determined previously.

(a) Sedimentation coefficient: $s_{20.w}$

Values for s were determined in five experiments. A mean value of $399 \pm 10 \times 10^{-13}$ s was obtained.

(b) Diffusion coefficient: $D_{20.w}$

The gel precipitin technique was used to calculate the diffusion coefficient.

$D_{20.w} = 0.73 \times 10^{-7}$ cm s^{-1}.

(c) Partial specific volume: $\bar{\nu}$

The partial specific volume was calculated by sedimentation of the virus in D_2O/H_2O.

$\bar{\nu} = 0.69 \pm 0.02$ ml g^{-1}.

(d) $\rho = 1.006$ g cm^{-3} for 0.06 M PB, and viscosity

$\eta = 1.026$ poises.

When the above data was inserted into equation (6) a mol. wt. of 43.4×10^6 was calculated for CPV of *H. armigera*.

7.5.2 Equilibrium Centrifugation

During sedimentation equilibrium a macromolecular substance dissolved in a single solvent or dilute buffer is sedimented until an equilibrium concentration gradient is established. The method permits direct determination of the weight-average mol. wt. The technique of sedimentation equilibrium takes longer to perform than determination of sedimentation rates; however, the distribution of particles at equilibrium is independent of their shapes and hydrations, so there is no need for data such as the diffusion coefficient.

Many viruses are too large for sedimentation equilibrium analysis, though Bancroft and Freifelder (1970) used the lower speed limit of 800 rpm to measure a mol. wt. of 49.4×10^6 for bacteriophage T7.

The following equation describes the distribution of an ideal single solute at equilibrium in a rotating centrifuge cell:

$$M = \frac{d \ln c}{d\, r^2} \cdot \frac{2\, RT}{(1-\bar{\nu}\rho)\omega^2} \qquad \ldots\ldots \quad (7)$$

where c = concentration of the solute.

The different notations have been defined in equations (2), (3) and (6).

The optics used were Rayleigh optics which are a convenient method of assessing concentration. Each fringe is effectively a graph that relates concentration on the y-axis to the distance from the centre of rotation on the x-axis. The concentration is measured as the fringe number relative to an unknown concentration at the meniscus. In the case of CPV, the mol. wt. was large enough so that the fringes became horizontal at the meniscus at equilibrium. Consequently, absolute virus concentration could be measured directly.

In order to measure the mol. wt. using equation (7), it is necessary to measure solute concentrations as a function of radius. A plot of $\ln c$ versus r^2 yields a straight line if the solute is both homogenous and thermodynamically ideal. However, if the solute is non-ideal or inhomogenous, a curve is obtained; the gradient at a particular point will give the apparent weight-average mol. wt. there. Plots that are concave upwards indicate polydispersity or aggregation and those that are concave downwards indicate non-ideality.

Method

Equilibrium ultracentrifugation was performed in a Beckman Model E analytical ultracentrifuge, using a heavy AN-J rotor to obtain maximum stability at low speeds, and a cell with a path length of 12 mm.

Purified CPV was exhaustively dialysed against buffer (0.03 M phosphate, 0.1 M KCl, pH 7.0) at 4°C. The two channels of a double-sector centerpiece were filled with 0.4 ml of virus solution and dialysate respectively. A speed of 800 rpm was used for the experiments and the solution was allowed to reach equilibrium during three days. The refrigerator unit, though not the temperature control, was operating during centrifugation, to prevent convection. Temperatures of 5^0C and 5.3^0C were recorded before and after the run. After equilibrium had been reached, the fringe pattern was photographed on Ilford G30 plates and measured as described above.

At the end of the run, the rotor was removed from the chamber, then shaken well to abolish the solute concentration gradient, returned to the chamber, and accelerated to 800 rpm. Photographs were taken to obtain a fringe baseline. The rotor was then accelerated to a higher speed (8000 rpm) and the sedimentation velocity pattern of the solute photographed with Schlieren optics to establish the condition of the virus at the end of the experiment. The pelleted virus was examined with the electron microscope to ascertain its condition after prolonged centrifugation.

Several additional measurements were necessary, including average rotor speed ascertained from readings on the revolution counter and times checked with a standard clock to within ±0.1 s. The buffer density was determined by pycnometry and confirmed from published tables. The horizontal magnification factor of the ultracentrifuge optical system was measured by means of the calibration cell.

Results

Model E ultracentrifuges, especially those with older drive units, are prone to rotor vibrations at low speeds. Precession of the rotor may be recognized by the characteristic distortion of Rayleigh interference fringes (Durham, 1972) and can also be visible to the eye as a side-to-side shaking of the image. Precession was present in two of the instruments used.

Precession caused practical problems during equilibrium sedimentation. A clear photographic image was not possible and the fringes faded out in areas. This made accurate measurements difficult and can be a significant source of uncertainty. Experiments to determine the $\bar{v}$ of the virus were also marred by effects of precession. In several runs only one image from one cell was observed or alternate images were missed and the boundaries blurred. Precession in these experiments was overcome by increasing the speed and using the heavy AN-J rotor.

As the sedimentation equilibrium experiments were not possible without overcoming precession effects, a simple lower bearing was designed to steady the axis of rotation of the rotor at low speed (Fig. 7.11). The mercury cup assembly was replaced by a bearing that consisted of a rubber stopper (41 mm in diameter) into which a rod of polytetrafluoroethylene (PTFE) (9 mm in diameter) was inserted. A hole was drilled in the upper surface of the PTFE to receive the blunted temperature-measuring needle at the bottom of the rotor. The bearing was correctly positioned with the aid of modelling clay which received the imprint of the contact needle when the chamber was slowly closed. The temperature regulation was not used during the experiment to reduce thermal convection, but the rotor came to thermal equilibrium with the chamber walls. With the aid of this device and in conjunction with a heavy AN-J rotor, fringe measurements were possible.

Equilibrium was reached within three days, although centrifugation was prolonged beyond this period. The method of overspeeding (Hexner *et al.*, 1961) caused too rapid sedimentation of the virus to be of use.

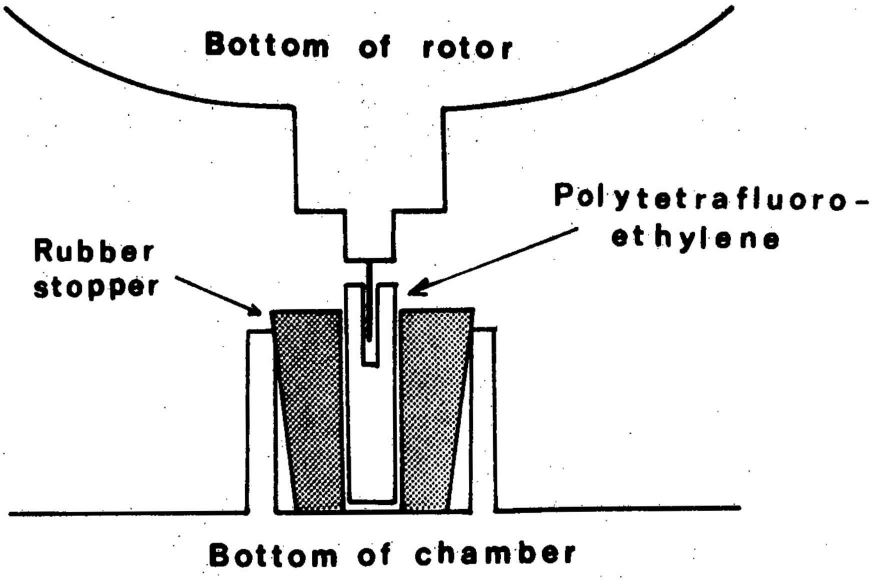


Fig. 7.11 Sectional drawing of antiprecession device which replaces the mercury cup thermistor contact in a Model E ultracentrifuge.

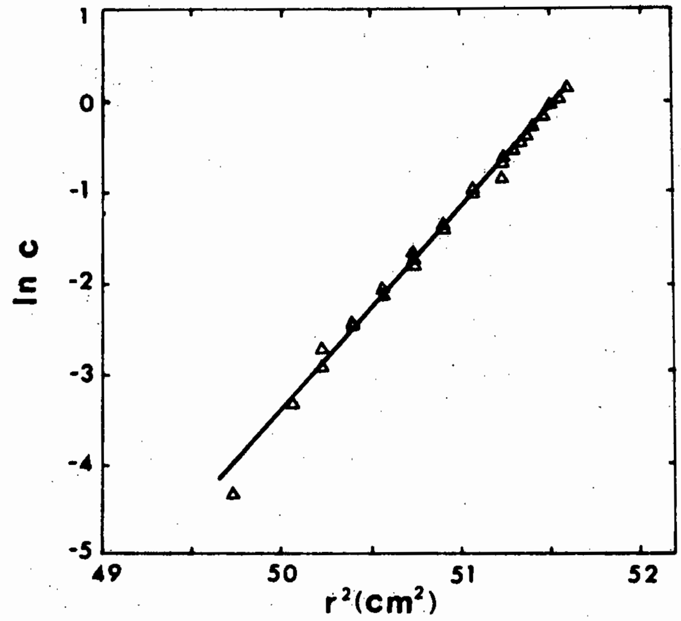


Fig. 7.12 A plot of ln c versus r^2 in equilibrium centrifugation of CPV_{A2} of *H. armigera*. Three separate interference fringes were measured . The sample volume was 0.4 ml in a cell with a path length of 12 mm. Centrifugation was at 800 rpm and 5°C; the buffer was 0.03 M phosphate, 0.1 M KCl, pH 7.0, *I* = 0.2.

The result from a successful equilibrium run is shown in Fig. 7.12 in which a plot of log of vertical fringe displacement against r^2 is shown. Vertical displacement values were obtained using three separate fringes on the photographic plate. A linear regression programme was used to calculate the slope for the line, and this value was substituted into equation (7) to calculate the weight-average mol. wt.

A molecular weight of 47.25×10^6 was calculated for the CPV_{A2} of *H. armigera*.

Virus particles examined by electron microscopy after a week of sedimentation equilibrium showed no apparent aggregation or damage.

7.5.3 Particle Counting

Particle counting is of value in confirming mol. wts. derived by other methods. In this method, low concentrations of virus may be used effectively. Electron micrographs of droplet patterns were used by Williams *et al.* (1951) to determine the particle weight of a known concentration of tobacco mosaic virus. They counted particles in a measured volume together with indicator particles of polystyrene latex whose concentration in the numbers of particles per unit volume had been calibrated. The method used here is that of Polson *et al.* (1970), in which the latex particles are replaced by haemocyanin of the whelk *Burnupena cincta.* These particles have a characteristic appearance and are easily distinguishable from virus particles in electron micrographs. They have the additional advantage of being more homogenous than latex particles.

In a mixture of virus and haemocyanin, a ratio of the number of virus particles to haemocyanin molecules is measured and from this the mol. wt. of the virus may be calculated.

$$M_V = \frac{C_V}{C_H} \times \frac{N_H}{N_V} \times M_H \qquad \ldots\ldots (8)$$

where C_V, C_H = concentrations of virus and haemocyanin respectively,

N_V, N_H = corresponding particle number, and

M_V, M_H = respective molecular weights.

Method

CPV_{A2} of *H. armigera* was purified as described. Haemocyanin was obtained from the mollusc *B. cincta* by heart puncture. The haemocyanin was suspended in PB, and contaminating material was removed by centrifugation at 6600*g* for 10 min in the Spinco 40 rotor. The haemocyanin was sedimented into a pellet at 59 000*g* for 1 h in the same rotor, and resuspended in a small amount of the buffer.

The concentrations of virus and haemocyanin samples were determined by ultracentrifugation using Rayleigh optics. The virus and haemocyanin samples were each dialysed against PB. A synthetic boundary cell was used at a rotational speed of 5200 rpm. At this speed sedimentation of the boundary and resultant radial dilution was minimal. The virus sample (0.15 ml) was placed in the one channel of the cell and the dialysate (0.45 ml) in the other. The initial speed was 8000 rpm until transfer of buffer was observed and the speed was then reduced to 5200 rpm. For the fractional fringe, the first photograph was taken as soon as fringes corresponding to overlaid buffer were sufficiently clear for measurement, usually about 2 min after buffer transfer had occurred. Further photographs were taken after 15 and 60 min for CPV and haemocyanin, respectively, and the number of whole fringes was counted using the Nikon microcomparator. The fringe count was used to calculate concentration using the formula:

$$J = \frac{\Delta n \ell}{\lambda} \qquad \ldots\ldots (9)$$

where J = number of fringes,

Δn = specific refractive index increment,

ℓ = path length, and

λ = wavelength of light.

Equal volumes (0.1 ml) of virus and haemocyanin suspensions in PB containing similar concentrations were mixed in a test tube. Distilled H_2O was added to reduce the concentration of salts present; this was done to avoid disrupting haemocyanin particles. Samples were examined in the electron microscope after negative staining (Fig. 7.13). Five fields were examined and photographed. The particle ratio was determined by counting the respective particles in photographic prints of each field.

Results

A typical field of a mixture of CPV and *B. cincta* haemocyanin is seen in Fig. 7.13. The numerical ratio of haemocyanin to CPV was ascertained. The number of particles counted for haemocyanin and CPV were 1313 and 180 respectively. By inserting these values together with the concentrations, determined as 0.227 mg cm^{-3} and 0.207 mg cm^{-3} for haemocyanin and CPV, respectively, and the previously determined

Fig. 7.13 Typical mixtures of CPV_{A2} of *H. armigera* and *B. cincta* haemocyanin. The number of particles of each was counted in fields similar to the lower figure. Note the large icosahedral CPV particle and the small, round or rectangular haemocyanin particle (x 120 000).

mol. wt. for haemocyanin of 7.9×10^6 (Polson *et al.*, 1970) in formula (8), a mol. wt. for CPV of 51.5×10^6 was calculated.

A Comparison of the Molecular Weights Determined

TABLE 7.6

THE MOLECULAR WEIGHT DETERMINATIONS ON THE CPV_{A2} OF *H. armigera* USING THREE DIFFERENT METHODS

Method	Molecular weight $\times 10^6$
Ultracentrifugation, s and D using Svedberg's equation (Section 7.5.1)	43.4
Equilibrium centrifugation using 'high speed' method (Section 7.5.2)	47.25
Particle counting (Section 7.5.3)	51.5
Mean value	47.4

7.6 DISCUSSION

The physical properties of the CPV and NPV of *H. armigera* and CPV of *N. cytherea cytherea* are summarized in Table 7.7. These values may be compared with those for other viruses in Table 1.1.

A comparison of some of the properties of nonoccluded and occluded CPV particles of *H. armigera* revealed no major differences. The buoyant densities for the free and occluded CPV virions were 1.44 and 1.45 g cm^{-3} respectively. This is similar to the finding by Payne and Kalmakoff (1974) that both types of CPV particles of *B. mori* were

TABLE 7.7

SUMMARY OF PHYSICAL PROPERTIES

Virus	Sedimentation coefficient S	Density in CsCl ($g\ cm^{-3}$)	Partial specific volume ($ml\ g^{-1}$)	Diffusion coefficient ($cm^2\ s^{-1}$) × 10^7	Particle weight (× 10^6)
CPV of *H. armigera*	399	1.444	0.69	0.73	47.4
CPV of *N. cytherea cytherea*	418				
NPV of *H. armigera*	1188	1.242			

Where no symbol occurs, no test was performed.

present in a density range between 1.43 - 1.48 g cm^{-3}. Studies by Lewandowski and Traynor (1972) and Lewandowski and Millward (1971) suggested that the range of particle density was due to differences in the RNA content of the viruses, the less dense virus lacking some of the smaller RNA segments. It is also possible that variation in density of virus particles results from loss or degradation of some of the structural polypeptides (Payne and Kalmakoff, 1974). Despite the surface effects observed in CPV of *H. armigera* after centrifugation in CsCl, the buoyant density measurements showed little variation.

The density determined in CsCl is the density of the virus in the hydrated state whereas $^1/_{\bar{v}}$ is the density of the unhydrated particle. CsCl is thought to decrease the hydration of viruses by osmotic effect.

There is usually a discrepancy between buoyant densities determined by isopycnic centrifugation in, for example, CsCl and the value for $^1/_{\bar{\nu}}$, but in the case of CPV_{A2} of *H. armigera* no such discrepancy was found.

Despite the difference in sedimentation coefficients for CPV and NPV of *H. armigera*, viz. 399 and 1188 S, complete separation of the particles by centrifugation alone was not possible as discussed in Section 3.5. There was no difference between free CPV virions and virions derived from polyhedra as shown by the determination of the sedimentation values of 384 S for both types of virions.

Several operational problems were encountered in performing analytical centrifugation and several Model E analytical centrifuges were used. At the low speeds required for the equilibrium centrifugation experiments, rotor vibration and precession became a serious problem. A lower bearing was devised to steady the axis of rotation and was successful in eliminating the precession sufficiently to permit measurements of the fringes by the Rayleigh interference method. A practical problem in determining the $\bar{\nu}$ was that at the low virus concentrations available, two samples had to be spun in the same rotor, when ultraviolet absorption optics were used. Since both the wedge centerpiece cells for interference optics and the multiplexer attachment were not available, a procedure was developed which used two single sector cells (with windows partially blacked out) for ultraviolet absorption optics and which allowed both samples to be photographed simultaneously.

Calculation of particle mol. wt. requires a knowledge of $\bar{\nu}$ which may be difficult to obtain. In studies in which a mol. wt. for CPV of *B. mori* was reported the $\bar{\nu}$ was not determined but values of 0.703 ml g^{-1} (Miura *et al.*, 1969) were assumed. With the low concentration of CPV_{A2}

of *H. armigera* available, methods commonly used could not be applied to measure $\bar{\nu}$.

Molecular weights and $\bar{\nu}$ have been determined by parallel sedimentation equilibrium experiments in D_2O/H_2O mixtures (Edelstein and Schachman, 1967). Although this method requires small quantities of material, it could not be applied to the CPV particle because of its large size. However the $\bar{\nu}$ could be measured by a sedimentation velocity method (Bellamy *et al.*, 1974). Sedimentation in D_2O/H_2O mixtures is difficult to apply to aggregating material but as CPV of *H. armigera* did not aggregate even after long periods of centrifugation, a modification of this method could be used.

Sedimentation rates in D_2O/H_2O mixtures enabled $\bar{\nu}$ to be measured rapidly and accurately and required a minimal amount of material. Bellamy *et al.* (1974) used a viscosity correction based on published values of $\eta D_2O/\eta H_2O$ mixtures. In the present study, the viscosity correction was calculated from data published by Klotz and Nagy (1963). From a plot of their viscosity data versus D_2O concentration, viscosity values for the D_2O concentrations used in this study were obtained. The correction factor of $k = 1.015$ in 100% D_2O for hydrogen-deuterium exchange (Martin *et al.*, 1959) is relatively constant for all proteins and could be applied to CPV of *H. armigera*. When the solvent contains D_2O at concentrations significantly below 100%, the correction factor is reduced proportionately.

From the sedimentation coefficient, partial specific volume, and diffusion coefficient, the mol. wt. of CPV of *H. armigera* could be estimated. This value was used to confirm the mol. wt. determined by equilibrium centrifugation and particle counting. Because of the low viral concentrations of the material available, the diffusion coefficient was determined by immuno-diffusion experiments. Past results obtained with this technique show good agreement with more conventional methods for determining the diffusion coefficient of viruses and other substances (Polson, 1958, 1976). The curve relating antigen dilution to band width was typically V-shaped (interrupted) (Polson, 1958, 1976; van Regenmortel, 1959). This indicates that the CPV antigen preparation was homogenous with regard to particle size as shown by Levitt and Polson (1964), whereas parabolic (continuous) curves are obtained with inhomogenous antigen preparations (Levitt and Polson, 1964; Juckes, 1974). The only precipitin bands to remain visible up to 21 days were those near optimal proportions. It is possible that the CPV antigen-antibody precipitate is soluble in the present of excess antigen.

For large viruses, the retardation effect of the gel becomes important but when the gel concentration is reduced to 0.1%, restriction in the free movement of particles as well as any agarose-virus interaction are reduced to a minimum (Wetter, 1967). The technique may have more inherent errors than other methods and would therefore contribute to the total error of the calculated mol. wt. as any deviation in D would be compounded in the molecular weight. Despite this, the mol. wt. of 43.4×10^6 derived by combination of s and D, is within the range of values reported for other viruses (Table 1.1).

The mol. wt. for the CPV of *H. armigera* obtained by the technique of particle counting, is higher than values calculated by other methods. Thomas (1961), in using this technique to determine the mol. wt. of *Tipula* iridescent virus, regarded the inclusion of contaminants in the determination of the concentration factor but uncounted on the electron micrographs as a possible source of error. This may account for the higher estimate (51.5×10^6) of the particle weight of CPV_{A2} of

H. armigera obtained with this technique. This difference was also reported by Marais (1970) for the β virus of *N. cytherea cytherea.*

The most accurate method of determining mol. wt., assuming an accurate value for $\bar{\nu}$ is sedimentation equilibrium. The value of 47.25×10^6 determined by this method falls between the values determined by the other two methods.

The average estimated mol. wt. of 47.4×10^6 for CPV_{A2} of *H. armigera* is slightly lower than the value generally assumed for *B. mori* CPV, 54×10^6, (Kalmakoff *et al.*, 1969). Their value was derived from sedimentation data, an assumed value for $\bar{\nu}$, and using the formula of Marvin and Hoffman-Berlin (1963). The CPV of *B. mori* is similar in many respects to that of *H. armigera*, the lower mol. wt. of the latter could be explained both by the absence of projections on CPV particles following purification, a possible contributory factor, and by the absence of a high-mol.-wt. RNA genome segment (Section 8.2.3). The total nucleic acid mol. wt. of CPV_{A2} is 12.47×10^6, whereas that of CPV_{A1} is 14.34×10^6. If it is assumed that the nucleic acid contributed approximately 27% to the particle weight of CPV_{A1} as for CPV_{A2}, the total particle weight of CPV_{A1} could be calculated to be 52.9×10^6. This value is closer to that of CPV of *B. mori* reported by Kalmakoff *et al.* (1969). On the same basis, the mol. wt. of the CPV_B of *H. armigera* and the CPV of *N. cytherea cytherea* would have mol. wts. of 52×10^6.

CHAPTER 8

CHARACTERIZATION OF NUCLEIC ACIDS

8.1 METHODS OF EXTRACTION AND ANALYSIS

8.1.1 Nucleic Acid Extraction

RNA Extraction:

The phenol sodium dodecyl sulphate (SDS) method of Scherrer and Darnell (1962) was used:

Buffer A, pH 5.2;

Buffer B like A, but containing SDS and bentonite;

Phenol reagent - phenol (saturated with water) containing 1% v/v of 8-hydroxyquinoline;

Chloroform with added 1% v/v of isoamyl alcohol, water-saturated to prevent any loss of aqueous sample.

A sedimented pellet of virus was resuspended in buffer A and an equal volume of buffer B was added. The mixture was kept at 37°C for 30 min. An equal volume of phenol reagent was added and rotormixed for 1 min, then placed in ice. The cooled mixture was centrifuged at 6000 rpm for 10 min in the Sorvall superspeed RC-2B centrifuge. The bottom phenol phase was removed and discarded. An equal amount of $CHCl_3$ reagent was added to the aqueous phase, which was mixed, cooled, and then centrifuged as above. The $CHCl_3$ extraction procedure was repeated three times, each time extracting the aqueous phase and interphase; the interphase was removed at the last extraction following centrifugation. Ethyl alcohol was added to the final aqueous phase (2-3 times its volume) and the preparation left at -15°C overnight to precipitate the nucleic acids, which were recovered by centrifugation at 10 000 rpm for 15 min in the same centrifuge.

DNA Extraction:

Reagent: 1 M Sodium perchlorate
0.01 M Tris, pH 8.2
1% SDS

The pelleted virus sample (NPV or GV) was resuspended in 3 ml of the extraction reagent and left at 37°C for 30 min. An equal volume of $CHCl_3$ (saturated as above) was added and mixed on a rotormixer for 1 min followed by centrifugation at 2500 rpm for 5 min. Subsequent $CHCl_3$ extractions and precipitations of DNA by ethyl alcohol were as described for RNA extraction.

8.1.2 Colour Reactions

The diphenylamine reaction (Dische, 1930) as modified by Burton (1956) was used for deoxyribose estimation. In this method, the reacting sugar is associated with the purine nucleotides. The orcinol procedure detects pentose sugars and was used for RNA estimation (Mejbaum, 1939).

The nucleic acids being studied were identified as DNA or RNA by means of the following tests:

(i) 1.2 ml of diphenylamine reagent (recrystallized with hexane) was added to 0.4 ml of extracted nucleic acids and 0.2 ml of 2 M perchloric acid.

(ii) A solution of nucleic acid extract was diluted to 1.5 ml with orcinol reagent (recrystallized using benzine) and boiled for 20 min.

Standard curves were drawn up using doubling dilutions of yeast tRNA in the orcinol reaction, and *Micrococcus lysodeikticus* DNA in the diphenylamine reaction. The colour produced by the unknown sample was expressed as a percentage of that produced by a quantity of standard giving an equivalent absorbance (after heat denaturation) at 260 nm prior to the reaction.

8.1.3 Buoyant Densities

Neutral caesium sulphate gradients were used to determine the buoyant densities of the nucleic acids.

Reagents: Caesium sulphate
Tris-HCl buffer, 0.001 M, pH 7.0

A saturated solution of Cs_2SO_4 in Tris-HCl buffer was mixed with the extracted nucleic acid sample dissolved in the same buffer to give for initial analysis a density of approximately 1.5 g cm^{-3}. The final volume was 5 ml. Centrifugation was at 110 000*g* for CPV and GV, or 83 000*g* for NPV, for 64 h at 25^{0}C in an SW 50.1 rotor. Tubes were pierced and the UV absorbance of the effluent was monitored at 254 nm. Fractions of 0.2 ml were collected, using an ISCO model UA 5 absorbance monitor and model 640 fraction collector. The density of each fraction was calculated from the refractive index at 25^{0}C, measured in a Hilger refractometer.

8.1.4 Melting Profiles

When double-stranded (ds) RNA or ds DNA are heated in dilute buffer, their optical densities show a sharp thermal transition due to strand separation (Montagnier and Sanders, 1963; Doty *et al.*, 1960).

The melting temperature at the midpoint of the transition is denoted as the T_m.

Thermal denaturation curves of extracted nucleic acid in dilutions of standard sodium citrate (SSC) buffer were determined using the SP 1800 ultraviolet spectrophotometer with an SP 876 series 2 temperature programme controller and heating unit. The wavelength was 258 nm and samples were heated at a rate of 1^0C/min. The increase in absorbance (hyperchromicity) was expressed as the ratio:

$$\frac{\text{absorbance at } T^0C}{\text{absorbance at } 30^0C} \quad \text{(Murant } et\ al.\text{, 1972)}$$

Melting profiles of CPV RNA were performed in 0.01 × SSC and that of NPV and GV in 0.1 × SSC to bring the thermal transitions into a convenient temperature range.

8.1.5 Enzyme Digestion Studies

Viral ds RNA resists digestion by pancreatic ribonuclease (RNase) when in concentrated salt solution but not in dilute salt solution, whereas single-stranded (ss) RNA is degraded in both.

Reagents: Enzyme-pancreatic RNase (protease free) Type Xl-A from Sigma Chemical Co., and deoxyribonuclease (DNase) from Miles Seravac Co.

(i) Solutions of extracted nucleic acids from CPV_{A2} in either 1 × SSC or in 0.01 × SSC were treated with 1 µg/ml of RNase for 30 min at 37^0C and absorbance was monitored on an SP 1800 spectrophotometer.

(ii) Nucleic acids prepared from NPV and GV were incubated with 10 µg/ml DNase for 30 min at 37^0C. The buffer was 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, volume 100 µl. The reaction was stopped when necessary by addition of 10 µl of 10 mM EDTA and 2% SDS.

8.1.6 Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) is used for fine resolution of nucleic acid species and is often used to determine molecular weights of nucleic acids, especially RNA segments in viruses with multipartite genomes.

Reagents: Acrylamide (BDH Analar) recrystallized with chloroform (Loening, 1967)
NN'methylenebisacrylamide (BDH Analar) recrystallized with acetone (Loening, 1967)
Buffer for electrophoresis, pH 7.5
NNN'N"-tetramethylethylenediamine (TEMED)(BDH Analar)
Ammonium persulphate (BDH Analar)

Polyacrylamide gel electrophoresis (PAGE) was performed as described by Loening (1969). A stock solution of 12% *m*/v acrylamide containing 0.6% *m*/v NN'methylenebisacrylamide to give 5% cross-linked gels was made and stored in the dark. Monomer solutions were made by dilution of this stock. Gel concentrations are described by the percentage of acrylamide present. The volumes of reagents for the range of gels used in this study are listed in Table 8.1. Details of the preparation of a 4% gel are given below:

13.33 ml	acrylamide stock, 12%
4.00 ml	10 × electrophoresis buffer
4.00 ml	glycerol
0.53 ml	TEMED
17.61 ml	water

The reagents were mixed and the mixture was degassed to remove oxygen which inhibits polymerization. 0.53 ml of a freshly prepared 10% *m*/v solution of ammonium persulphate in degassed water was added to the degassed mixture. 5 ml of this mixture was immediately poured into each perspex tube (0.6 × 10 cm) with one sealed end. Degassed buffer

TABLE 8.1

PREPARATION OF GELS CONTAINING 2.0-4.0% ACRYLAMIDE (5% CROSS-LINKED)

Gel conc. %	12% Acryl-amide Stock	Electro-phorosis buffer 10X	Glycerol	10% TEMED	10% Ammonium persulphate	H_2O
2.0	6.67	4.00	4.00	0.26	0.26	24.81
2.1	7.00	4.00	4.00	0.28	0.28	24.44
2.2	7.33	4.00	4.00	0.29	0.29	24.09
2.3	7.67	4.00	4.00	0.30	0.30	23.73
2.4	8.00	4.00	4.00	0.32	0.32	23.36
2.5	8.33	4.00	4.00	0.33	0.33	23.01
2.6	8.67	4.00	4.00	0.34	0.34	22.65
2.7	9.00	4.00	4.00	0.36	0.36	22.28
2.8	9.33	4.00	4.00	0.37	0.37	21.93
2.9	9.67	4.00	4.00	0.38	0.38	21.57
3.0	10.00	4.00	4.00	0.40	0.40	21.20
3.1	10.33	4.00	4.00	0.41	0.41	20.85
3.2	10.67	4.00	4.00	0.42	0.42	20.49
3.3	11.00	4.00	4.00	0.44	0.44	20.12
3.4	11.33	4.00	4.00	0.45	0.45	19.77
3.5	11.67	4.00	4.00	0.46	0.46	19.41
3.6	12.00	4.00	4.00	0.48	0.48	19.04
3.7	12.33	4.00	4.00	0.49	0.49	18.69
3.8	12.67	4.00	4.00	0.50	0.50	18.33
3.9	13.00	4.00	4.00	0.51	0.51	17.98
4.0	13.33	4.00	4.00	0.53	0.55	17.61

Final volume in each case = 40 ml, equivalent to 8 gels, allowing 5 ml per gel.

These quantities were scaled down according to the number of gels of any one concentration required.

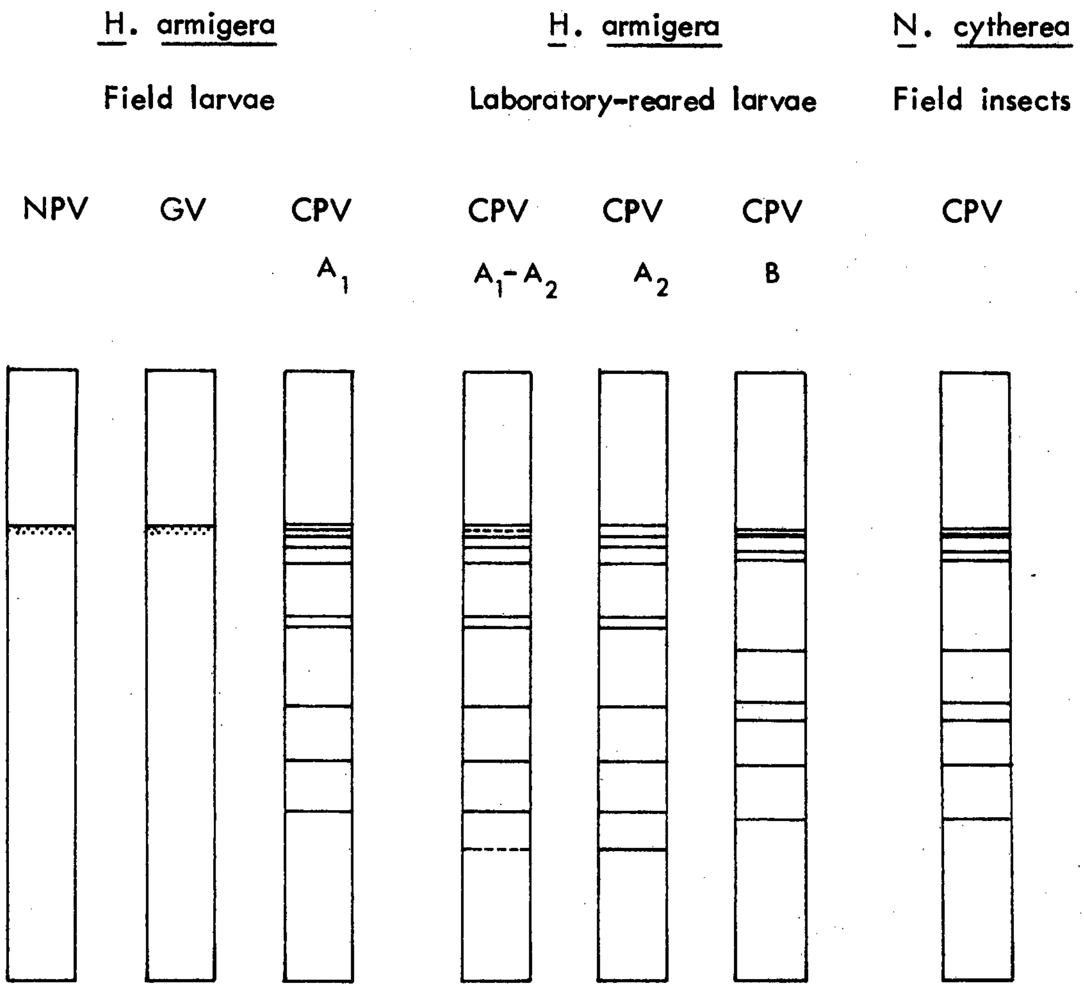
diluted 1 : 10 was layered gently on to the polymerizing mixture to ensure a flat surface and prevent the access of oxygen. The mixture was left at 4^0C to polymerize and used the following day. Tubes were cleared at 100 *V* for 1 h before use. 20 to 50 µl of a solution of electrophoresis buffer diluted with water (1 : 4) and containing 20% glycerol was added to dissolve the sample, which was then layered onto the gel surface.

Electrophoresis was performed at 20^0C and 12.5 *V*/cm of gel (approximately 50 amps per gel) for 5-6 h. The use of constant conditions avoids voltage and temperature-dependent mobility variations (Fisher and Dingman, 1971). Electrophoresis was performed in an apparatus that included cooling units.

After electrophoresis, gels were measured and removed from the tubes. They were placed in a 12-cm silica spectrophotometric cell, covered with electrophoresis buffer, and scanned at 265 nm in a Varian model 635 spectrophotometer. The distance moved by each species through the gel was calculated by multiplying the distance (mm) on the trace from the origin, by the ratio of the total gel length to the total chart trace length. Gels to be stained were placed in 2% toluidine blue overnight, then destained either electrophoretically or in 15% acetic acid. Alternatively, gels were stained with ethidium bromide (0.5 µg/ml in electrophoresis buffer).

When appropriate, the gels were sliced with a Mickle Laboratories gel slicer. For ^{32}P estimation, slices of gel were placed in vials containing 5 ml of water, incubated at 37^0C for 3 h, and then counted by Cerenkov radiation in a Beckman scintillation spectrometer.

KEY* TO NOMENCLATURE WITH REPRESENTATIVE VIRAL NUCLEIC ACID POLYACRYLAMIDE GEL ELECTROPHORETIC PROFILES



8.2 RESULTS

The preceding key* will assist in correlating the strains isolated in this study with their laboratory reference subscripts.

8.2.1 Identification of nucleic acid structure

The nature of the pentose sugar was determined by the orcinol and diphenylamine reactions. The results obtained are summarized in Table 8.2.

The nucleic acid from CPV gave a typical green colour with the orcinol reaction, and the absorbance at 660 nm was similar to that given by an equivalent quantity of yeast tRNA. Both NPV and GV nucleic acids gave colour intensities in the diphenylamine reaction, proportional to the intensity given by an equivalent quantity of *M. lysodeikticus* DNA.

The buoyant densities of the various nucleic acids are shown together with the buoyant density of *M. lysodeikticus* DNA in Table 8.2 and graphically in Fig. 8.1B. The *M. lysodeikticus* DNA was included as an internal check on the accuracy of the estimations, and its value of 1.43 ± 0.01 g cm^{-3} is within the range of reported values. The buoyant density of 1.60 obtained for the nucleic acid of CPV is characteristic of RNA and is closer to the values typical of ds RNA than of ss RNA (1.61 for reovirus, Shatkin, 1965). The buoyant densities of NPV and GV nucleic acids are typical of DNA.

Thermal denaturation resulted in a marked hyperchromicity over a narrow temperature range for each virus nucleic acid (Fig. 8.1A; Table 8.2), indicating ds configurations. The melting curve for CPV nucleic acid showed a slight initial rise at lower temperature, comparable to that observed by Payne and Tinsley (1974) for *Nymphalis io* CPV RNA. The reason for this is unknown.

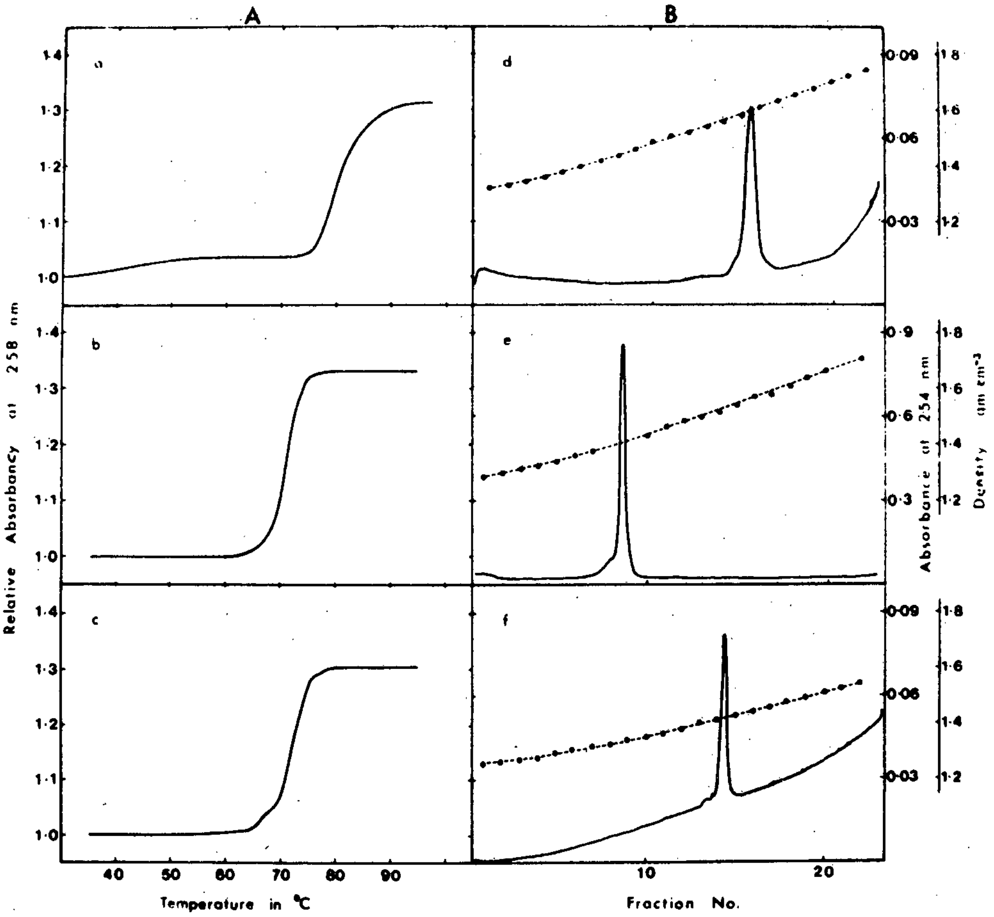


Fig. 8.1 A. Thermal denaturation of nucleic acids (a) CPV, (b) GV, and (c) NPV; CPV in 0.01 x SSC, GV and NPV in 0.1 x SSC.

B. Caesium sulphate equilibrium centrifugation of (d) CPV, (e) GV, and (f) NPV nucleic acids.

A_{254} nm —— ; density g cm^{-3} ●···●

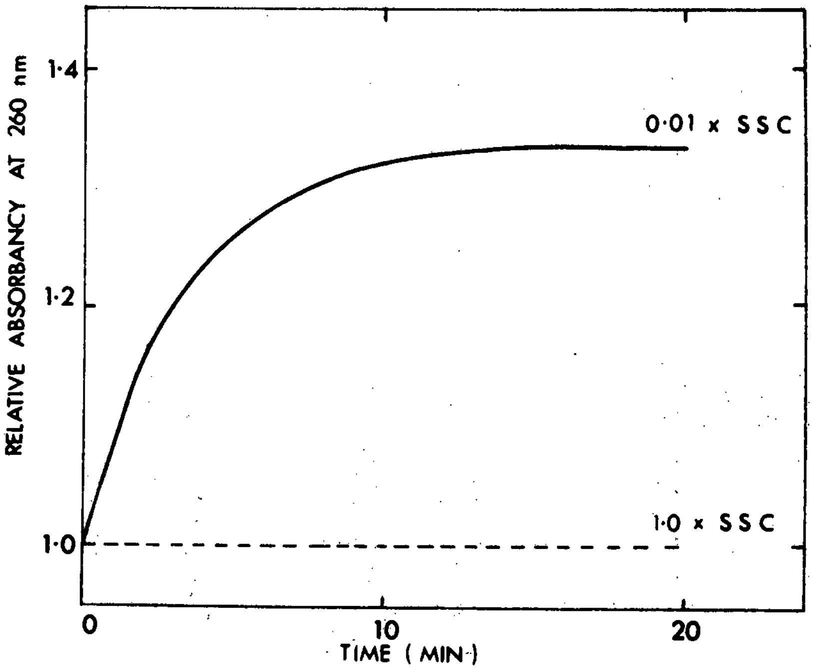


Fig. 8.2 Effect of RNase digestion on CPV_{A2} RNA

Viral RNA was incubated with 1 µg/ml RNase in either 1.0 x SSC or 0.01 x SSC at 37°C and absorbancy followed at 260 nm.

TABLE 8.2

Nucleic acids	Colour Reactions		Melting temperature (^0C)	Buoyant density in Cs_2SO_4 (g cm^{-3})
	Orcinol (%)	Diphenylamine (%)		
CPV	105	-	80	1.60
GV	-	96	72	1.41
NPV	-	95	71	1.42
M. lysodeikticus DNA	-		-	1.43

— = not tested

Sensitivity to digestion by RNase or DNase was assessed by incubating the nucleic acid with enzyme at 37^{0}C and then observing hyperchromicity. The hyperchromicity of nucleic acid from CPV was only 1.01 (A_{258nm} at 30 min; A_{258nm} at 0 min) with 1 μg/ml RNase in 1 × SSC but was 1.33 with 1 μg/ml RNase in 0.01 × SSC; with a T 1/2 of 3 min in the latter case (Fig. 8.2). Digestion of GV and NPV nucleic acids with 10 μg/ml DNase for 30 min at 37^{0}C gave hyperchromicities of 1.29 and 1.32, respectively.

These studies showed that CPV contained ds RNA and that GV and NPV, ds DNA.

The viral genomes were characterized further by polyacrylamide gel electrophoresis. Typical results are shown in Fig. 8.3. NPV and GV both gave a single, slightly diffuse band with low electrophoretic mobility identical to that of HeLa DNA. CPV_{A2} RNA, however, showed ten discrete bands and prior digestion of the CPV RNA with either pancreatic RNase in 1 × SSC or DNase (as described) did not change its profile.

Additional evidence relating to the structure of the nucleic acids

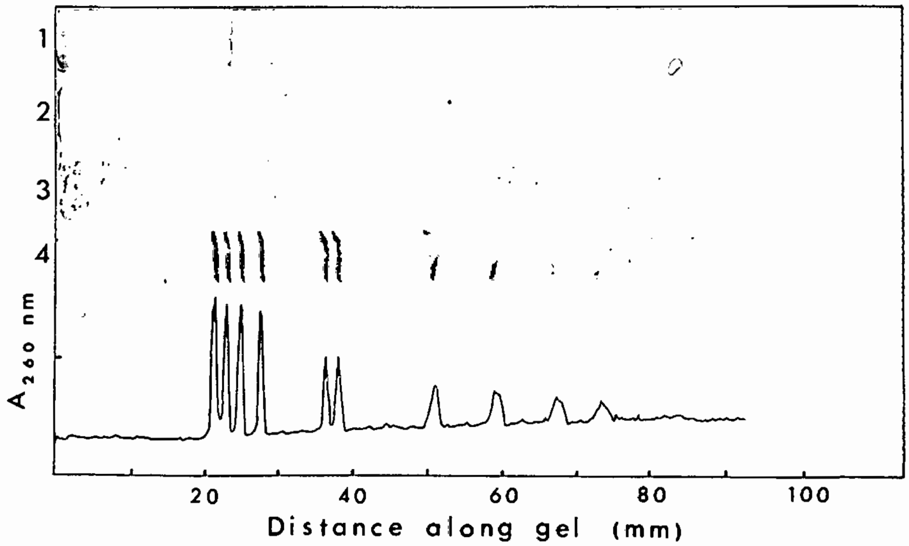


Fig. 8.3 Electrophoretic comparison of (1) HeLa DNA, with nucleic acids extracted from (2) granulosis virus, (3) NPV, and (4) CPV (Photograph and $A_{254\ nm}$ scan); 2.6% gels, run for 4 h at 10 V/cm (1)-(3), Run together; (4), on a separate occasion. Electrophoresis from left to right.

TABLE 8.3

RELATIVE MOLECULAR WEIGHTS AND ELECTROPHORETIC MOBILITIES OF CPV$_{A2}$ RNA GENOME SEGMENTS ON 2.6% POLYACRYLAMIDE GELS

Segment No.	Relative Mol. wt. expressed as % of total ^{32}P cpm	S.D.	Mobility relative to slowest segment 1	S.D.	Absolute Mobility* Cm^2 volt^{-1} sec^{-1} × 10^5
1	20.75	0.80	1.00	-	1.92
2	17.56	0.61	1.06	0.01	2.04
3	14.62	0.30	1.15	0.01	2.20
4	12.89	0.56	1.26	0.02	2.42
5	8.98	0.91	1.67	0.05	3.19
6	8.37	0.60	1.74	0.05	3.33
7	5.82	0.27	2.30	0.07	4.41
8	4.76	0.26	2.66	0.08	5.10
9	3.82	0.19	3.01	0.09	5.76
10	3.28	0.11	3.25	0.10	6.23

* Determined by measuring mobility relative to DNA co-electrophoresed with CPV$_{A2}$ RNA on a 2.6% gel using previously determined values for DNA absolute electrophoretic mobility at 2.6% acrylamide concentration (Harley *et al.*, 1973).

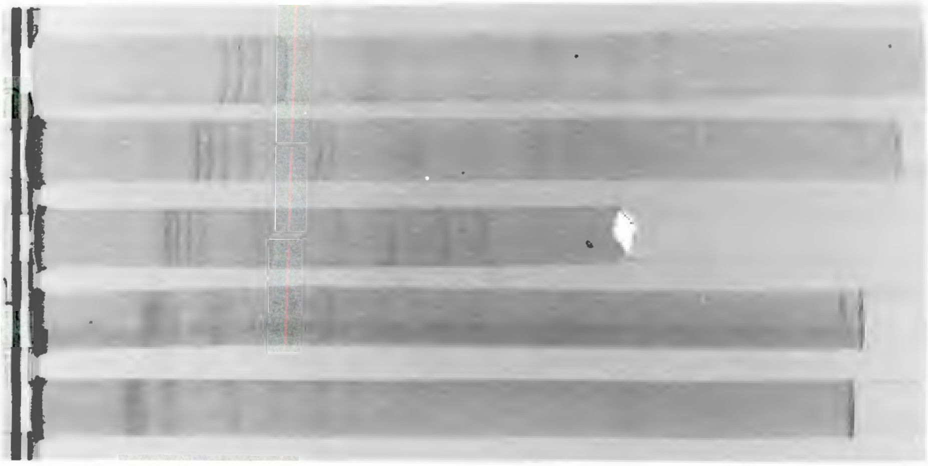


Fig. 8.4 Gel range analysis of CPV_{A2} nucleic acids. The gel concentrations ranged from 2.3 - 4.0% (top to bottom). Electrophoresis is from left to right.

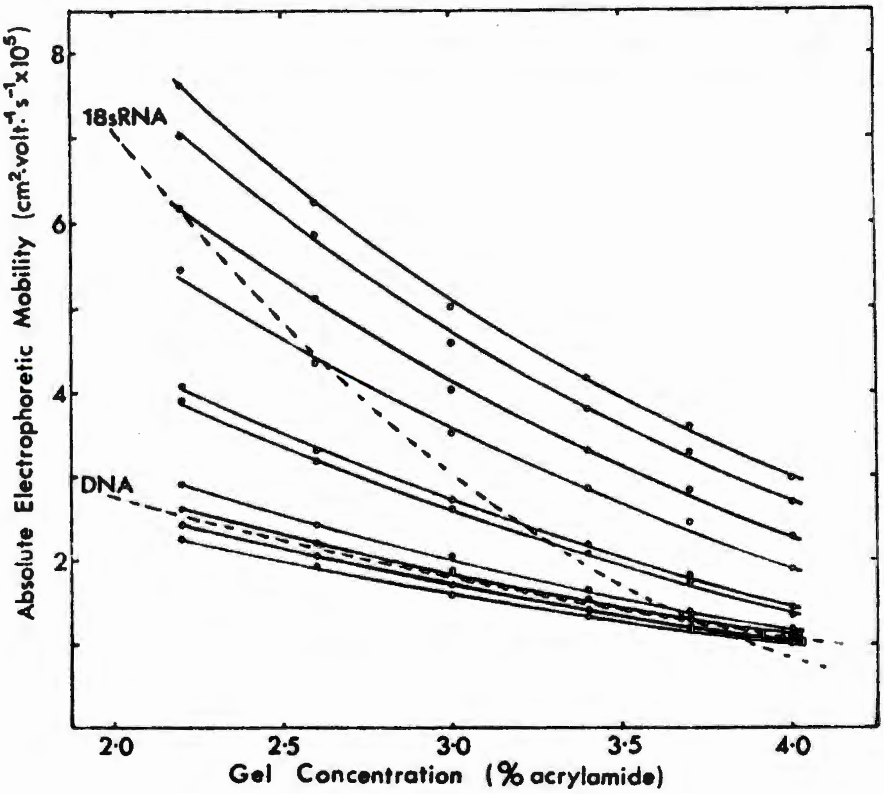


Fig. 8.5 Variation in absolute electrophoretic mobility of CPV nucleic acids with gel acrylamide concentration ——, CPV species; ----, curves for HeLa 18 S ribosomal RNA and HeLa DNA shown for comparison. Each point represents the mean of at least three different observations at that gel concentration.

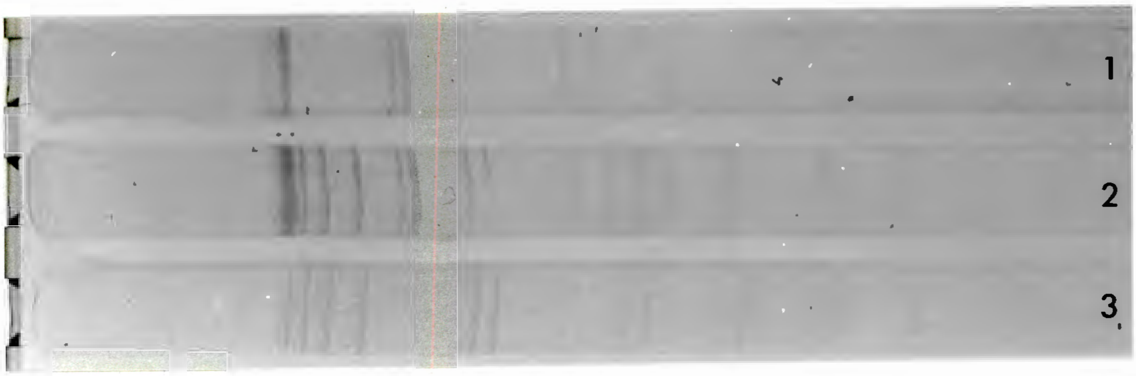


Fig. 8.6. Co-electrophoresis of CPV_{A2} RNA and reovirus type 3 RNA.

1. Reovirus RNA
2. Reovirus RNA and CPV_{A2} RNA
3. CPV_{A2} RNA

Electrophoresis was for 5 h at 10 *V*/cm on 2.6% gels. Electrophoresis was from left to right.

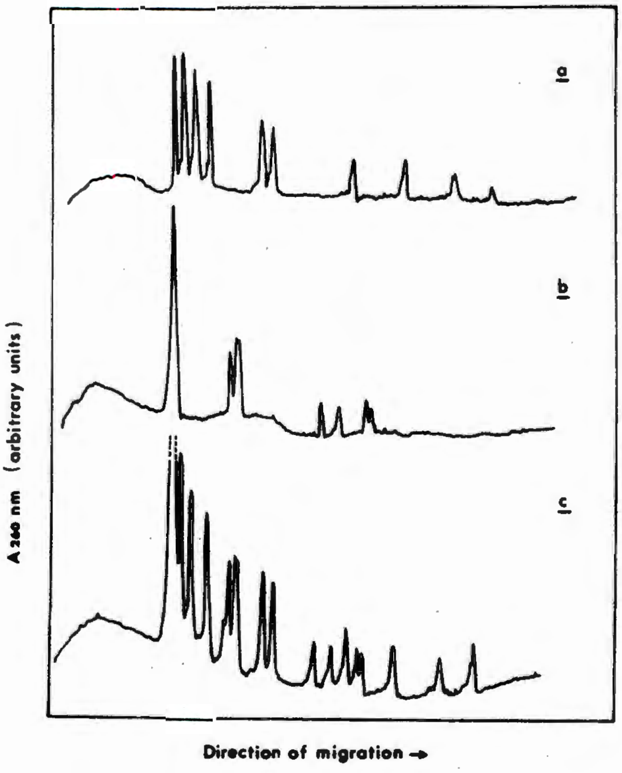


Fig. 8.7 A_{260nm} scans of the above gels.

a. CPV_{A2} RNA b. Reovirus type 3 RNA c. Reovirus and CPV_{A2} RNA was provided by electrophoresis on a range of gels of differing concentrations. Results are shown in Figs. 8.4 and 8.5. All ten genome segments of CPV_{A2} RNA show the flat curve characteristic of ds linear species (Harley *et al.*, 1973). NPV and GV RNA at various gel concentrations showed the flat curve characteristic of a ds linear species, and on co-electrophoresis of either with HeLa cell DNA, there was no separation of the viral nucleic acid from HeLa DNA.

The ds RNA genome segments of CPV_{A2} were remarkably well separated on PAGE, unlike segments of reovirus RNA. This made the CPV_{A2} virus uniquely suitable for a study of other multicomponent ds genomes.

8.2.2 Molecular Weight Determination of CPV_{A2} RNA Genome Segments

Table 8.3*lists the electrophoretic mobilities of CPV_{A2} RNA segments run on 2.6% gels under similar conditions relative to the mobility of segment 1 (the slowest migrating genome segment). Relative electrophoretic mobility was converted to absolute by co-electrophoresing CPV_{A2} RNA with DNA of known absolute electrophoretic mobility (Harley *et al.*, 1973). Reovirus type 3 RNA was used as a molecular weight (mol. wt.) standard, since the average mol. wts. of its three main genome groupings have been determined as summarized by Bellamy *et al.* (1967) and Shatkin *et al.* (1968). Reovirus RNA and CPV_{A2} RNA were co-electrophoresed and Figs. 8.6 and 8.7 show comparative photographs and scans. The electrophoretic mobility of the CPV_{A2} RNA was measured relative to that of the reovirus bands. Using the known mol wt. of either 'L' or 'M' genome segments of reovirus type 3 RNA (Bellamy *et al.*, 1967; Shatkin *et al.*, 1968) mol. wt. values could be assigned to specific absolute electrophoretic mobilities on 2.6% gels. The range

*page 195

of mobilities for the reovirus type 3 'S' group was too wide to be used.

The relationship between mol. wt. and electrophoretic mobility was established by analysing CPV_{A2} RNA generally labelled with ^{32}P, on a number of gels, choosing optimal conditions of electrophoresis to separate either the slow-moving or fast-moving RNA species. Two typical electropherograms are shown in Fig. 8.8. The amount of ^{32}P in each peak from a total of 12 gels was measured and yielded the relative mol. wt. of each segment.

The plot of log relative mol. wt. versus electrophoretic mobility deviates markedly from linearity at higher mol. wts. (Fig. 8.9). The 'M' group of reovirus ds RNA segments has an average mobility conveniently positioned midway between that of the CPV bands 4 and 5 and provides a point on the curve of known mol. wt. 1.3×10^6. In this way the relative mol. wt. scale can be calibrated in absolute mol. wt. units.

Using the curve calibrated in this way by reo 'M' RNA with respect to mol. wt., a value of 2.4×10^6 for the larger reovirus band 'L' was read off the curve. This shows good agreement with the values of 2.3×10^6 (Bellamy *et al.*, 1967) and 2.5×10^6 (Shatkin *et al.*, 1968) for the 'L' group of reovirus ds RNA derived by physico-chemical methods. This helps to confirm the non-linearity of the plot.

Further confirmatory evidence that the plot of log mol wt. versus electrophoretic mobility for ds RNA deviates from linearity at high mol. wts. was obtained by a study of the replicative form (RF) RNA of Foot and Mouth disease virus (FMDV). This ds RNA is of higher mol. wt. than any of the reovirus or CPV_{A2} RNA segments examined, with mol. wt. (for the ss RNA) estimated to be 2.6×10^6 (Talbot and Brown, 1972). The mol. wt. of the replicative form would be double this value. The electrophoretic characteristics of FMDV RF are typical of a fully ds

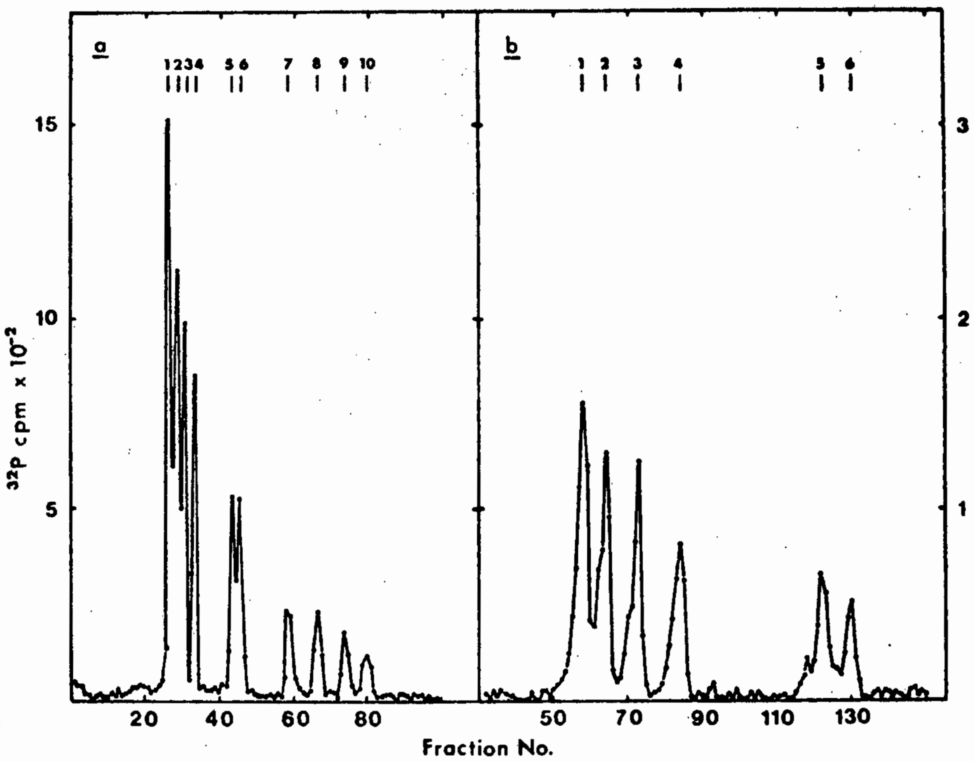


Fig. 8.8 Polyacrylamide gel electropherograms of ^{32}P-labelled CPV$_{A2}$ RNA electrophoresed for either a) 4 h or b) 8 h at 10 V/cm. Electrophoresis was from right to left.

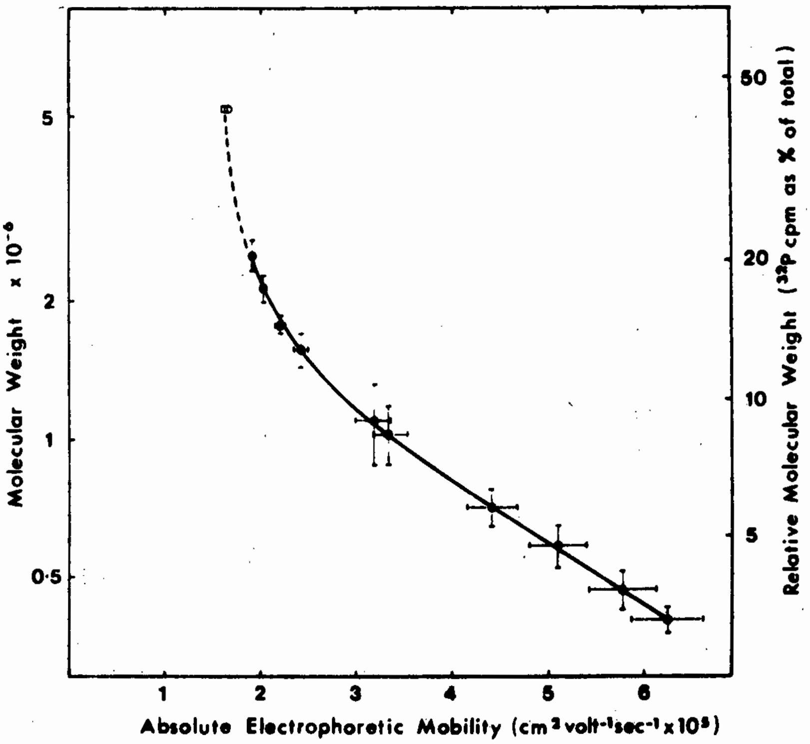


Fig. 8.9 Plot of log molecular weight versus absolute electrophoretic mobility for the ten ds RNA genome segments of CPV ● and the RF RNA of FMDV ▭. Relative mol. wt. of any segment is expressed as its percentage of the total ^{32}P in all ten genome segments. The bars express the units of 2 x S.D. on each axis. Absolute mol. wt. was derived by calibration with reovirus as described.

linear structure. Published values for the mobility of FMDV RF relative to DNA (0.74) on 2.6% gels were used (Harley *et al.*, 1973). When the mol. wt. and electrophoretic mobility values for FMDV RF were included in Fig. 8.9, the plot fell on a predictable extrapolation of the curve relating CPV_{A2} RNA mol. wt. to electrophoretic mobility.

The mol. wt. values of CPV RNA genome segments were read from the calibrated curve and are listed in Table 8.4.

TABLE 8.4

MOLECULAR WEIGHTS OF CPV_{A2} RNA GENOME SEGMENTS

Segment No.	Mol. wt. $\times 10^{-6}$	S.D. $\times 10^{-6}$
1	2.55	0.11
2	2.15	0.09
3	1.80	0.04
4	1.58	0.08
5	1.10	0.13
6	1.03	0.08
7	0.71	0.04
8	0.58	0.04
9	0.47	0.03
10	0.40	0.02
Total	12.37	

8.2.3 Alteration in PAGE Profiles of CPV RNA

Three separate isolates of CPV were prepared from larvae of *H. armigera* collected from infected fields of either lucerne, tomatoes, maize or lupin in the Western Cape. The fields were 50 kilometres apart and the three isolates were made in consecutive years. Each virus preparation was then passaged in laboratory-reared *H. armigera* larvae. Each larva was infected with approximately 3 × 10^5 polyhedra. Yields were measured by counting polyhedra prepared from excised midguts, using a Bright-Line haemocytometer. CPV was prepared and nucleic acid extracted as described previously.

Each initial isolate (100 to 300 larvae) from a given field gave only small yields of CPV; two of the isolates contained mixtures of NPV and CPV and required preliminary separation of the CPV from the NPV as described. RNA extracted either from the free virions or from polyhedra gave ten bands of ds RNA when analysed on PAGE as illustrated in Fig. 8.10(a). This was different from the picture obtained from CPV prepared after several passages through laboratory-reared larvae (Fig. 8.10(b)). The only difference between the two profiles was the absence in (b) of the second slowest migrating band present in (a) and its replacement in (b) by a band migrating faster than any of the others (Fig. 8.10). All the bands seen in (a), including the additional slow moving band absent from (b), were resistant to digestion with either DNase 1 or RNase 1 at high ionic strength, confirming that all the bands were ds RNA. There was no change in relative electrophoretic mobility of these bands when electrophoresed on gels ranging in concentrations from 2.0 to 4.4%. The electrophoretic patterns were designated A1 for the initial pattern, and A2 for the later pattern (see key). In Fig. 8.10(c) is shown an intermediate stage, where the second slowest migrating band of CPV_{A1} is markedly reduced in intensity as compared with neighbouring bands. In addition, the fast-moving band characteristic of CPV_{A2} is now visible.

The change from the CPV_{A1} to the CPV_{A2} pattern occurred very rapidly. After one or two passages of the initial viral isolate (using either free virions or polyhedra for infection) in laboratory-

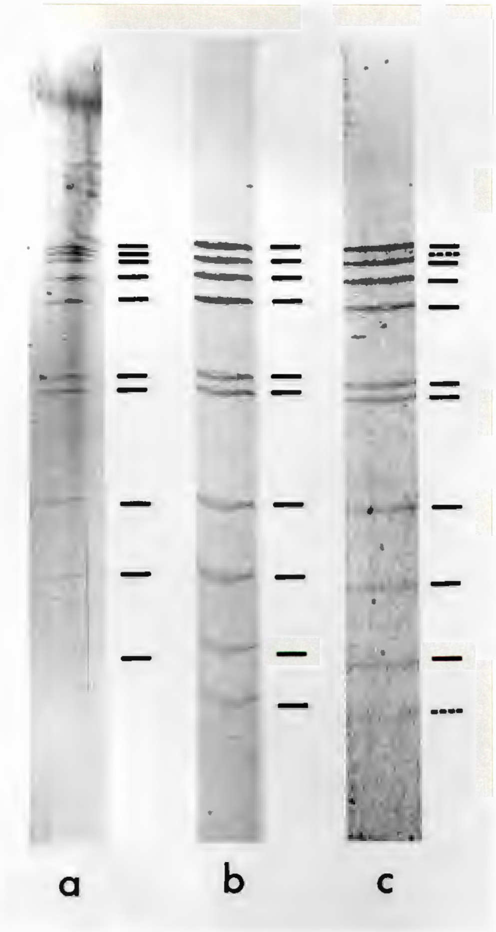


Fig. 8.10

Polyacrylamide gel electropherograms of CPV RNA.

(a) An initial isolate from the field

(b) After several passages of a field isolate through laboratory-reared larvae

(c) After one passage of a field isolate through laboratory-reared larvae

Electrophoresis was for 4 h at 10 *V*/cm on 4.0% gels. Migration was from top to bottom. Gels were stained with toluidine blue, and bands are shown in diagrammatic form on the right of each gel.

Fig. 8.11

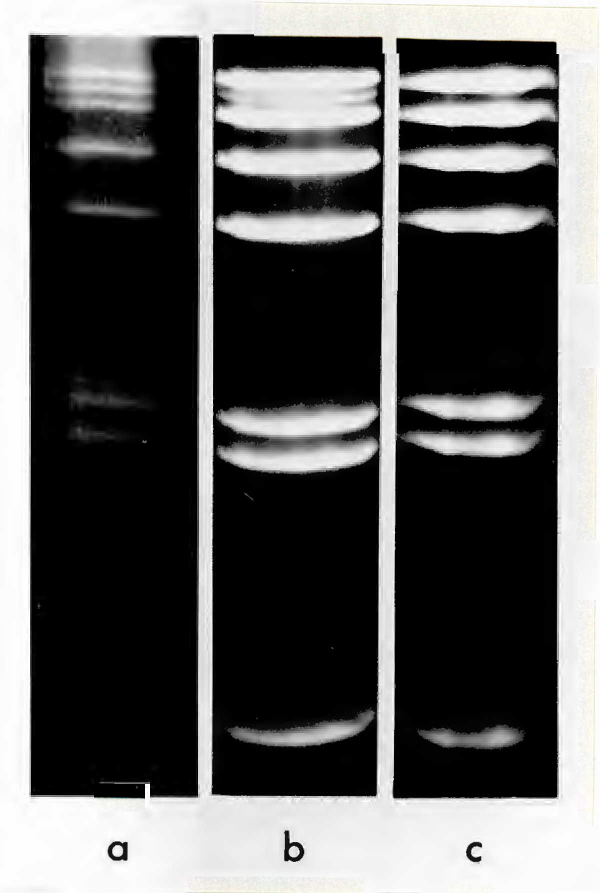
Polyacrylamide gel electropherograms of RNA from two successive passages of an isolate of CPV.

(a) Original pattern before passage

(b) After first passage

(c) After second passage

Enlargement to show the higher molecular weight species. Electrophoresis was for 5 h at 10 *V*/cm on 4.0%. Gels were then stained by immersion in a 0.5 μg/ml solution of ethidium bromide in electrophoresis buffer, and photographed under 340 nm UV illumination.



reared larvae, the change in the gel electrophoretic pattern of the extracted RNA was complete. Figure 8.11 illustrates the progressive disappearance of the second genome segment of the CPV_{A1} isolate over the course of two successive passages. In all gel electrophoretic analyses of RNA preparations showing the transitional picture there was no alteration or splitting of any of the other bands.

There was no variation in mobility of nine of the bands and on this basis similar mol. wt. values were assigned to each of the CPV_{A1} genome segments corresponding to the CPV_{A2} genome segments. Since the mol. wt. of CPV_{A2} has now been determined by comparative electrophoretic mobility (Fig. 8.9), the mol. wt. of the additional slow band in CPV_{A1} could be calculated to be 2.3×10^6, making the total mol. wt. of the RNA of CPV_{A1} equal to 14.27×10^6, compared with 12.37×10^6 for CPV_{A2} (Table 8.4).

Using these mol. wt. values, molar proportions of the high-mol.-wt. segment (CPV_{A1}) and the low-mol.-wt. segment (CPV_{A2}) compared to genome segments 1-9, were determined from densitometry either of gels scanned at 258 nm or of photographic negatives of ethidium bromide stained gels. Equivalent values were given by each method and the results are shown in Table 8.5. The high-mol.-wt. segment was present in equimolar proportion to the other segments in CPV_{A1}. Similarly, equimolar quantities of the fast-moving segment were found in CPV_{A2}. Estimates of the minimum detectable quantities of these bands gave an upper limit for CPV_{A1} in CPV_{A2} preparations of less than 1%. Similarly, the upper limit for CPV_{A2} in the original isolate of CPV_{A1} was estimated to be less than 6%. The sum of the molar proportions of the high- and low-mol.-wt. segments were equal to 1.04 and 1.01 respectively in two intermediate patterns corresponding to two separate passages of CPV_{A1} isolates.

TABLE 8.5

MOLAR PROPORTIONS OF THE VARIABLE SEGMENTS

Genome type	Molar proportion ± S.E.M.*	
	Slow-moving band	Fast-moving band
A_1	1.03 ± 0.03	< 0.06
Intermediate 1	0.49 ± 0.03	0.55 ± 0.03
Intermediate 2**	0.21 ± 0.02	0.80 ± 0.08
A_2	< 0.01	1.01 ± 0.05

*S.E.M. = Standard error of mean

**Illustrated in Fig. 8.11

The yields of polyhedra recovered from laboratory-bred larvae represented an increase of 100 to 1000-fold compared with the original inocula and gave no indication of falling off with increasing passage up to seven sequential passages through laboratory-reared larvae. The altered genome pattern of CPV_{A2} also remained stable for at least five subequent passages.

Laboratory conditions were altered to see whether the change from CPV_{A1} to CPV_{A2} could be prevented or delayed; alterations included maintaining infected larvae at ambient rather than at a constant temperature of 28^0C; alteration of diet, substituting tomatoes for the artificial medium normally used, and exposure to natural lighting conditions, as opposed to rearing them in an incubator. Since some isolates were from mixed infections with NPV, some larvae were co-infected with CPV_A2 and NPV. The genome profile did not revert to the CPV_{A1} type, nor was the CPV_{A1} pattern retained in CPV_{A1}-infected larvae under any of the above conditions.

8.2.4 Presence of a CPV with a different RNA PAGE profile

A fresh batch of control *H. armigera* pupae for rearing was obtained from the PPRI. Before infectivity experiments, the first batch of larvae was examined for the presence of any latent virus. They contained small amounts of another CPV that were morphologically the same as CPV_{A1} and CPV_{A2} and had a buoyant density similar to that of CPV_{A2}. However, nucleic acid studies on PAGE revealed a genome segment pattern quite unlike that of CPV_{A1} and CPV_{A2}, but very similar to or identical with that of the CPV of a different, unrelated moth, *Nudaurelia cytherea cytherea* (Fig. 2.2). Subsequent studies confirmed that the genome profiles of the two viruses on PAGE were identical (Fig. 8.12). The virus isolated from *H. armigera* was named CPV_B, to differentiate it from CPV_{A1} and CPV_{A2} previously described.

After propagation in *H. armigera* larvae, free and occluded CPV_B virions gave the same genome profile on PAGE. Since the mol wt. of CPV_{A2} has now been accurately determined, it provides an appropriate standard to determine the mol. wt. of CPV_B by the corrected comparative gel electrophoretic method. Hence nucleic acids from a mixture of CPV_{A2} and CPV_B were electrophoresed on the same gel as described in Section 8.2.2, and from the relative mobilities of their genome segments the mol. wts. of those of CPV_B were estimated (Fig. 8.13). These are presented in Table 8.6.

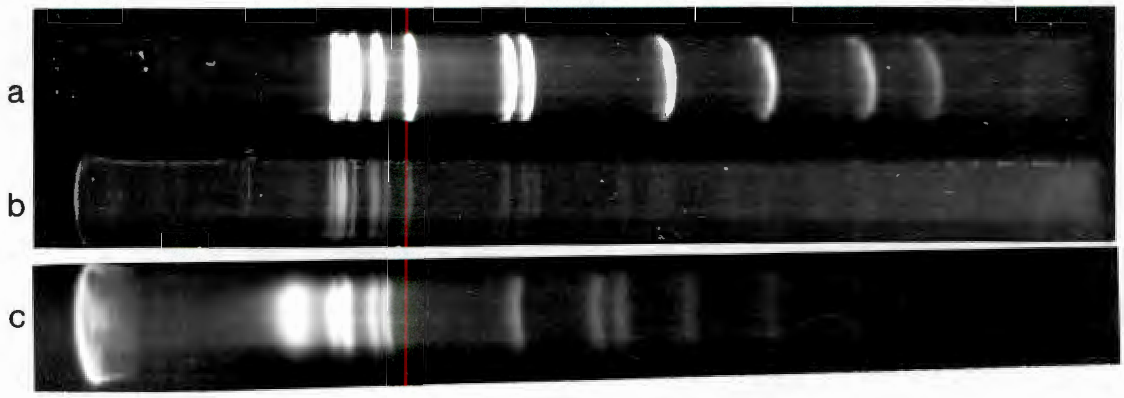


Fig. 8.12 Polyacrylamide gel electrophoretic profiles of CPV viruses isolated from *H. armigera* and *N. cytherea cytherea.*

(a) Intermediate stage of CPV_{A1} - CPV_{A2} of *H. armigera*

(b) Mixture of CPV_{A2} and CPV_{B} of *H. armigera* electrophoresed together on the same gel

(c) CPV of *N. cytherea cytherea* showing the same gel profile as CPV_{B} of *H. armigera* (separate run)

Electrophoresis is from left to right. The gel concentration is 4%. Gels were stained with ethidium bromide.

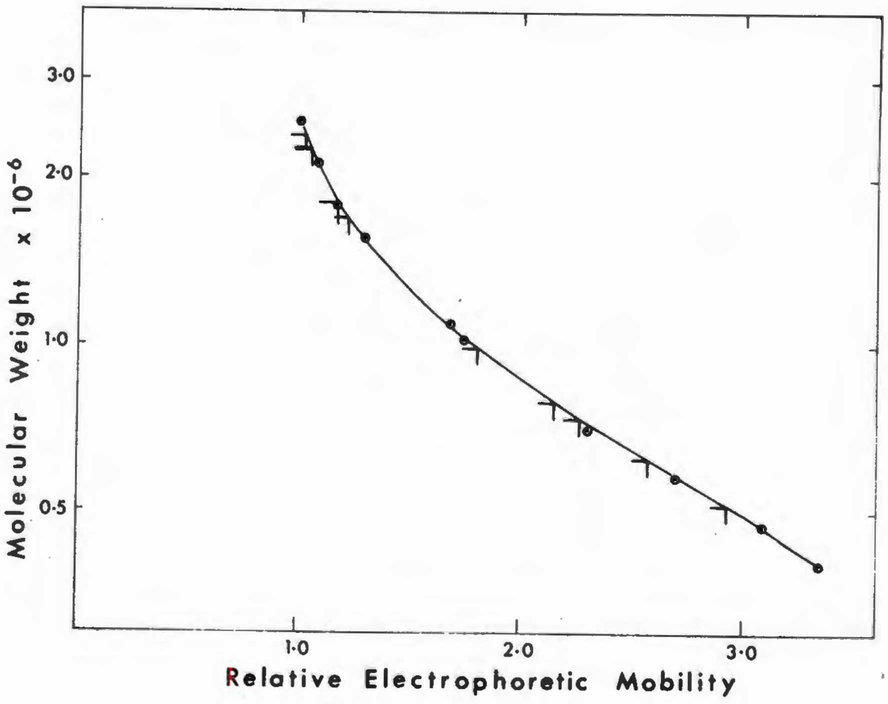


Fig. 8.13 Determination of molecular weight of CPV_{B} RNA by comparative PAGE with CPV_{A2}. Electrophoretic mobilities derived from co-electrophoretic analysis as illustrated in Fig. 8.12. Mol. wts. of marker CPV_{A2} derived from Table 8.4.

● - CPV_{A2} data points

⌝ - Relative electrophoretic mobility of CPV_{B}

TABLE 8.6

MOLECULAR WEIGHTS OF CPV$_B$ RNA GENOME SEGMENTS

Segment number	Molecular weights × 10^{-6}
1	2.38
2	2.25
3	2.24
4	1.80
5	1.70
6	0.99
7	0.79
8	0.74
9	0.62
10	0.51
TOTAL:	14.02

8.3 DISCUSSION

The nucleic acids of the viruses infecting *H. armigera* were analysed in detail in order to provide further data for their identification. Colorimetric measurements and buoyant densities showed that NPV and GV contained DNA whereas CPV contained RNA. Results of thermal denaturation studies, which distinguish between ss and ds configurations, showed that NPV and GV contained ds DNA while CPV contained ds RNA. These conformations were confirmed by enzyme digestion studies.

The viral genome was further characterized by polyacrylamide gel electrophoresis (PAGE) in order to provide more conformational data, to resolve genome components and to determine their molecular weights where possible. Both NPV and GV DNA behaved as ds linear DNA on gels of differing concentrations and CPV$_{A2}$ RNA behaved as ds linear RNA that was resolved into ten discrete bands. Prior digestion of CPV_{A2} RNA with either pancreatic RNase or DNase did not change its profile, confirming that each band was ds RNA. The plot of electrophoretic mobility versus gel concentration exploits the fact that separation of nucleic acid species by PAGE in non-denaturing buffers is based both on molecular size and on conformational properties. Electrophoretic mobility varies with gel concentration but the degree to which it does so is a characteristic of the conformational properties of a species (Fisher and Dingman, 1971). This characteristic differs for ss as compared with ds species, and for linear as compared with circular species (Harley *et al.*, 1973).

NPV and GV DNA both migrated as a single band on PAGE, indicating a mol. wt. greater than about 5×10^6 since at high mol. wt. values the mobility of ds linear DNA becomes independent of mol. wt. (Loening, 1967; Harley *et al.*, 1973). Only at lower mol. wt. values, such as those obtained by cleavage with restriction endonucleases (Danna and Nathans, 1971), are adequate separations of linear ds DNA achieved. Detection of high mol. wt. circular DNA (Summers and Anderson, 1972, 1973) would not be expected with the extraction procedure used in this study, since DNA with a molecular weight greater than 10^8 would be sheared.

The separation of CPV RNA genome segments into discrete bands on PAGE is characteristic of CPV (Miura *et al.*, 1968; Kalmakoff *et al.*, 1969; Lewandowski and Millward, 1971; Hayashi and Krywienczyk, 1972; Payne and Tinsley, 1974 and Payne and Rivers, 1976) reovirus (Shatkin *et al.*, 1968; Bellamy *et al.*, 1967; Miura *et al.*, 1968) and also of other members of the Reoviridae. The electrophoretic mobility of the ds RNA components at a specific gel concentration is unique for each virus and provides a profile of the genome for comparative studies.

Molecular weight values of viral RNA are important as an estimate of the size of the viral genome and are of interest in the structural arrangement of the virus. The mol. wt. of *H. armigera* CPV$_{A2}$ ds RNA could be determined by comparative gel electrophoresis since the ds RNA CPV$_{A2}$ genome segments separate well on PAGE unlike those of other CPVs. Adequate separation is necessary for accurate measurement of each genome segment by ^{32}P incorporation. In reoviruses and similar viruses such as CPV, the genome segments have been found to be present in equimolar proportion (Millward and Graham, 1970; Lewandowski and Millward, 1971). Thus measurement of the mol. wts. of the ds RNA genome segments by ^{32}P incorporation is a valid and practical method. By this approach the predicted non-linear relationship of the plot of log mol. wt. versus electrophoretic mobility (Section 1.8) for ds RNA was defined. The plot in Fig. 8.9 illustrates how mol. wt. determination based on a straight-line plot and using reovirus RNA or other ds RNA species as standards will be subject to error at high mol. wt. values. The shape of the curve suggests that the electrophoretic mobility of ds RNA reaches a limiting value as the mol. wt. increases. This behaviour is similar to that of ds DNA (Harley *et al.*, 1973). Ideally ds RNA species of even higher mol. wt. than those described are necessary to confirm this phenomenon. This postulated lower limiting value for the electrophoretic mobility of ds linear RNA gives rise to a 'forbidden region' at the proximal end of gels electrophoresed under set conditions where no ds linear species should be found. Some other ds RNA species have been recently examined in this context (Bozarth and Harley, 1976).

Variation in gel concentration between 2.4 and 4.0% does not affect the non-linear nature of the curve relating log mol. wt. to electrophoretic mobility above mol. wt. values of 10^6, since relative electrophoretic mobility does not vary significantly within this range. The technique of gel range analysis was previously used to identify the number of strands in a RNA species by Harley *et al.* (1973), who showed that the slopes of curves differed markedly for ss and ds RNA. They found that ss and ds RNA behaved like the corresponding RNA species and also that ds circular species had a different slope.

On the basis of characterization of their nucleic acids, NPV and GV meet the criteria for classification in the genus Baculovirus (Wildy, 1971), and CPV, in the Reoviridae - the genomes of the latter should consist of 10-12 molecules of ds RNA with a combined mol. wt. of approximately 15 × 10^6 daltons (Wildy, 1971; Joklik, 1974).

Several kinds of genome alterations have been reported in the Reoviridae. Stable alterations consisting of minor differences in the electrophoretic mobility of some RNA segments have been observed with different serotypes of reovirus (Shatkin *et al.*, 1968) and a rotavirus (Kalica *et al.*, 1976). Loss of specific genome segments in defective virus particles has also been observed after high multiplicity passage in tissue culture. Nonoyama *et al.* (1970) and Nonoyama and Graham (1970) found that serial passage of the Dearing strain of reovirus at medium to high multiplicity produced particles that lacked the L1 genome RNA segment, and had nine rather than ten ds RNA segments. These particles resembled the virions morphologically but were not infectious on their own. Schuerch *et al.* (1974) have shown that selected temperature-sensitive mutants lose certain genome segments during replication. Other studies have shown the deletion of genome segments from defective particles (Reddy and Black, 1974).

Prolonged passage (up to 24 years) of wound tumour virus (WTV) in the plant host without passage through the insect vector (i.e. only one of its two alternate hosts) resulted in gradual loss and deletion of some genome segments. Reddy and Black (1974) observed loss of transmissibility by the vector and partial or complete loss of infectivity for vector cell monolayers. Genomes of the mutants showed different PAGE profiles and the authors postulated that these morphological changes in the genome segments may be a cause of the loss of transmissibility or infectivity. Twenty-eight different mutations were detected on PAGE.

During the present study, a change in the genome composition of CPV was observed on laboratory passage. The segment with the second-highest-mol.-wt. was replaced by a fast moving low-mol.-wt. segment as seen on PAGE; the phenomenon was reproducible despite the variety of geographical locations from which field larvae were obtained and the period over which samples were collected. These observations do not appear to represent an example of a defective virus, since several cycles of replication continued in the host insect and viral yields even appeared to be enhanced. The change in genome pattern from CPV_{A1} to CPV_{A2} is rapid and reproducible. There appear to be two possible explanations for this phenomenon:

1. that each of the three isolates consisted of a mixture of two CPVs, as has already been described for the CPVs of *Arctia caja* (Payne, 1976) and perhaps *Malacosoma disstria* (Hayashi and Krywienczyk, 1972; Payne, 1976) and that laboratory conditions favoured CPV_{A2} at the expense of CPV_{A1}. This explanation would imply that CPV_{A2} was present in each of the three initial isolates in very low proportions relative to CPV_{A1}, and that favourable conditions for selection of CPV_{A2} in preference to CPV_{A1} must

exist under the conditions in which larvae were reared and infected; and

2. a mutation may have occurred in the second genome segment of CPV_{A1} - probably a deletion with retention of a smaller fragment, approximately 1/6 the size of the original, manifest as band 10 of CPV_{A2}. It is difficult to conceive, however, what selective conditions would enable such a phenomenon to occur on three separate occasions with such rapidity. On the other hand, the similarity of nine of the ten genome segments of the CPV_{A1} and CPV_{A2} suggests that even if two separate viruses are present in the initial isolate, CPV_{A2} has arisen at some stage as a mutant from CPV_{A1}.

A sample of *H. armigera* larvae containing CPV_{A1} was sent to Dr. Payne (Unit of Invertebrate Virology, Oxford) who showed that the PAGE RNA profile was very similar to that of the CPV of *Trichoplusia ni* but with minor differences in mobility of some of the genome segments on co-electrophoresis (Payne and Rivers, 1976). The low yields of CPV_{A1} in field isolates has precluded more extensive studies such as hybridisation to show relationships between CPVs with similar genomic groupings.

The phenomenon observed in laboratory passage of *H. armigera* CPV differs from phenomena described for both reovirus and WTV. In the former case, bands disappeared in a sporadic manner that was not reproducible, and in WTV, the disappearance of the segments was frequently not complete, took a long period of time to manifest and was only partially reproducible (Reddy and Black, 1974).

Many different CPVs have been reported in the literature, usually referred to by the name of their host on the early assumption that insect viruses were host specific. Several studies have described exceptions to this theory (Bergold, 1958; Aizawa, 1963; Smith, 1963). Opinions have differed as to whether the finding of different viruses in insects is due to genuine transmission from another host or to stimulation of a latent virus as a result of stress. Neilson (1964) achieved cross-transmission from one insect to another and back-cross transmission with a CPV from *Vanessa cardui* to eleven out of eighteen species of Lepidoptera and two of Hymenoptera. It appears that the same virus can infect more than one species.

The present study is another example of how two different CPVs can infect and replicate in the same host. The virus particles of CPV_A and CPV_B were the same morphologically, but they are readily distinguished from each other on PAGE of the ds RNA genome segments.

Previously nucleic acid and protein data had been reported for only 3 CPVs (with the exception of the CPVs in the present study). Payne and Rivers (1976) have recently published data on 33 CPV isolates, with nucleic acid studies being one of the main criteria. With these data to hand, characterization of CPVs using PAGE should provide one of the best means of classifying the many reported CPV isolates.

Such biochemical information for CPVs together with serological studies should be able to determine whether a newly isolated CPV is in fact a new virus or a previously described virus.

CHAPTER 9

DETERMINATION OF THE MOLECULAR WEIGHTS OF PROTEINS USING POLYACRYLAMIDE GEL ELECTROPHORESIS

Molecular weights were determined by gel electrophoresis (Ornstein, 1964; Davis, 1964) using the Tris-glycine discontinuous buffer system of high resolving power recommended by Laemmli (1970). A semi-micro slab gel apparatus was modified from those described by Adams *et al.* (1969) and Studier (1972).

9.1 METHOD

A stock solution of Cyanogum 41 (30% *m*/v) in distilled water was purified by the addition of activated charcoal, thorough stirring, and filtration through Whatman No. 1 filter paper. All solutions were prepared in distilled water unless otherwise stated.

The gel-forming apparatus was set up as illustrated (Fig. 9.1).

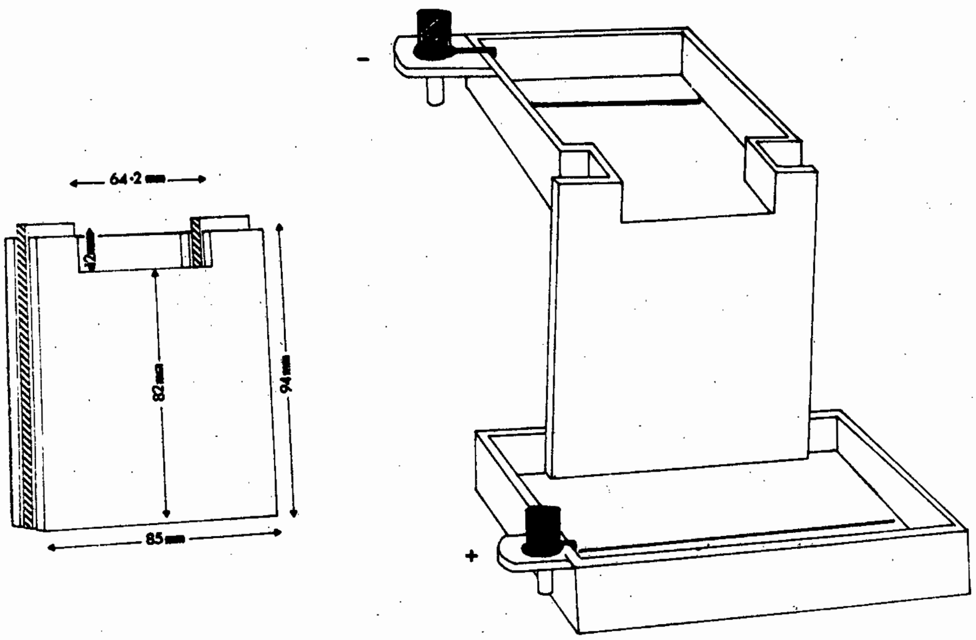


Fig. 9.1 Apparatus used to prepare polyacrylamide gel (left) and for electrophoresis (right).

Two glass plates, separated by perspex spacers on three sides were sealed with hot 2% agar (*m*/v) and held together with bulldog clips. A 5-20% gradient gel was prepared as follows:

TABLE 9.1

REAGENTS USED FOR PREPARATION OF GRADIENT GEL SOLUTIONS

Reagents	5% (ml)	20% (ml)	Stacking gel (3%) (ml)
Cyanogum 41 (30%)	1.0	3.3	0.75
Buffer A, pH 8.8	1.0	1.0	-
Buffer B, pH 6.8	-	-	1.0
Riboflavin (0.004%)	0.05	0.05	0.05
TEMED (N,N,N',N'-tetramethylethylenediamine)	0.01	0.01	0.01
SDS (10%)	0.05	0.05	0.05
Glycerol (87%)	-	0.5	-
Distilled water to give a final volume of	5.0	5.0	5.0

The solutions were degassed under vacuum and placed in a gradient mixer, the solution of higher concentration being inserted in the well with the outlet to the mould. After the gradient was poured, a small volume of distilled water was carefully layered over it to ensure a flat gel surface after polymerization. The mould was exposed to light and the gel allowed to polymerize for 1 to 1½ h. After polymerization, the water was decanted and the gel surface rinsed with the prepared degassed stacking gel: 2 to 3 ml of stacking gel was applied above the polymerized gel. The sample wells were formed by placing a perspex slotted template above the resolving gel, within the stacking gel solution. Polymerization was continued for a further 3 h. The template was withdrawn and a syringe used to remove any liquid in the sample channels. The perspex spacer at the bottom of the slab mould was removed and the mould clamped on the electrophoresis apparatus, and electrophoresis buffer added to the top and bottom compartments of the apparatus.

Protein samples were prepared as follows:

(i) Viral protein was prepared by adding sample buffer, together with SDS and mercaptoethanol, to purified viral material (20-30μg of protein). The sample (15-20μl) was boiled for 2 min and used immediately.

(ii) Purified polyhedra were treated as viral protein but the ratio of buffer to sample was increased.

(iii) Polyhedral protein was prepared by dissolving purified polyhedra in alkaline buffer (0.02 M Na_2CO_3, 0.05 M NaCl), pH 10.6 for periods of 5, 15, and 30 min. The pH was adjusted with HCl to pH 5 to precipitate the polyhedral protein. The precipitate was collected by low-speed centrifugation and washed several times with 0.1 M phosphate buffer pH 6.0. The final precipitate was dissolved in sample buffer before electrophoresis.

Protein samples (3-15μl) were applied to each of the sample channels with a Hamilton microsyringe. Electrophoresis was conducted at 5 mA for 5 h. After electrophoresis, the gel was removed from the mould, fixed in 12.5% (*m*/v) TCA for 1-2 h, washed with destaining solution for 1 h, and stained in Coomassie blue (Section 4.1.3) for 45 min. The gel was now destained with destaining solution until it was clear. Destained gels were stored in 7% CH_3COOH. To detect carbohydrate, the gels were stained using Clarke's method (1964). After being fixed in TCA as above, gels were washed with distilled water and 7% CH_3COOH, followed by immersion for 1 h in 1% periodic acid in 3% CH_3COOH and 1 h in Schiff's reagent. Gels were kept in the dark for the last two steps and destained in 1% (*m*/v) sodium metabisulphite and stored in this solution.

9.2 RESULTS

Protein molecular weights (mol. wt.) of virions and polyhedra were determined by electrophoresis of known protein standards together with samples. Commercially available proteins were used as mol. wt. markers with the exception of γ-globulin obtained from the Microbiology Department, UCT. The mol. wt. of standards used covered the entire range of unknown proteins.

	Molecular weight
γ-globulin (anti-TMV) prepared in rabbits	150 000
Transferrin (Sigma Chem. Co.)	90 000
Bovine serum albumin (Miles Seravac Co.)	67 000
Ovalbumin (Miles Seravac Co.)	43 000
Carbonic anhydrase (Sigma Chem. Co.)	29 000
Myoglobin (Miles Seravac Co.)	17 000
Cytochrome C (Miles Seravac Co.)	11 700

Gels were prepared as described, and standards (3-6μg) and samples, previously solubilized, were applied to the same gel. Following electrophoresis and staining, the migration distances into the resolving gel were measured for all proteins. A graph was plotted of this distance against the $\log_{10}$ of the molecular weight. A reproducible plot for mol. wt. values between 11 000 to 150 000 was obtained (Fig. 9.2), from which the mol. wts. of the viral proteins could be calculated. An example of the PAGE profile of the resolved viral and polyhedral proteins,

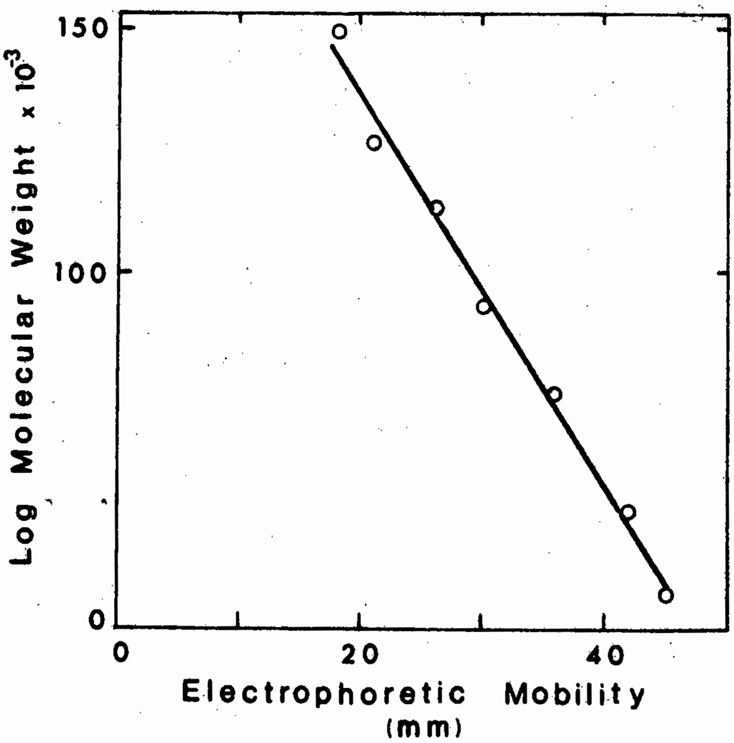


Fig. 9.2 Molecular weight determinations of proteins based on electrophoretic migration of a range of marker species. Log mol. wt. was plotted against the distance migrated for the following markers: from top to bottom, globulin, transferrin, bovine serum albumin, ovalbumin, carbonic anhydrase, myoglobin, and cytochrome C. A least squares method was used to obtain the best straight line to fit the data points.

together with that of some of the standards, is shown in Figs. 9.3 and 9.4. The separation and resolution obtained on gradients were superior to that originally obtained on single concentration gels. Molecular weights were calculated from the mobility values on gradient gels. Similar values were obtained for samples electrophoresed on bis-acrylamide gradient gels.

The proteins of CPV_{A2} polyhedra ranged in mol. wt. from 11 800 to 145 000. The major protein had an approximate mol. wt. of 28 700 (15 measurements) and was consistently present in large quantities (Fig. 9.3). Proteins usually present in smaller quantities had molecular weights of approximately 95 000; 51 000; 25 400; 17 200 and 11 800.

Other bands with mol. wts. of 145 000 and 14 200 were frequently visible. Addition of urea did not alter the PAGE profile.

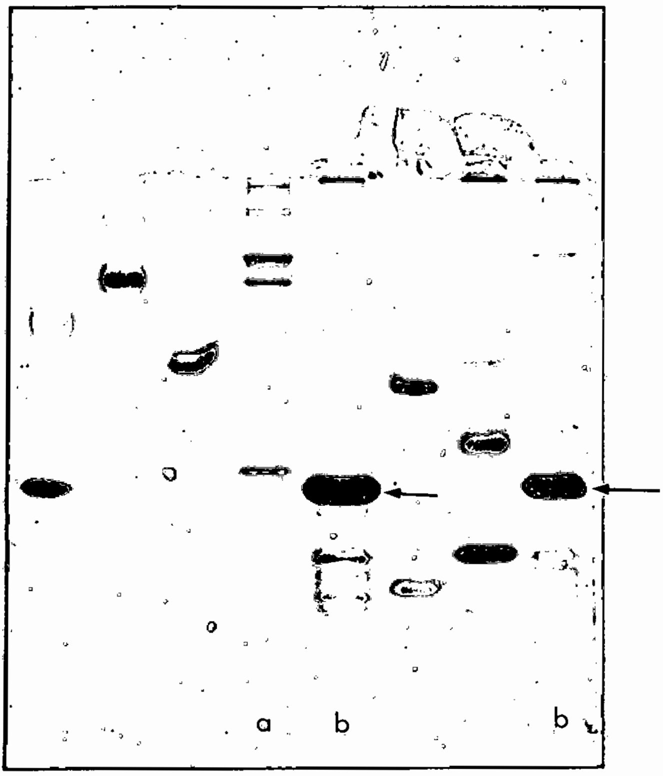
The proteins of CPV_{A2} virions ranged from 13 400 to 163 000. The major protein had a mol. wt. of approximately 32 400 (14 measurements) and was consistently present. Proteins present in most tubes but in lesser amounts had mol. wts. of approximately 163 000; 139 000; 37 000; 28 600 and 16 900. A minor band with approximate mol. wt. of 125 000 was sometimes visible as were bands with mol. wts. greater than 160 000. It was observed that when bands in the mol. wt. range of 32 000 - 160 000 were present, low mol. wt. bands were either absent or present in only trace amounts (Fig. 9.3).

Some polyhedral (P) and viral (V) proteins were similar in mol. wt., i.e. P 145 000, V 139 000; P 28 700, V 28 600; P 17 200, and V 16 900.

The mol. wts. of several of the polyhedral and viral proteins were similar to that of some estimated gene products (Table 9.3), but the remainder do not appear to be derived from specific genome segments.

Fig. 9.3 Polyacrylamide gel electrophoresis of: standards; *H. armigera* CPV$_{A2}$ virions (a), and polyhedra (b) on a gradient gel. The pattern of CPV$_{A2}$ virion shows the high-mol.-wt. bands. The major polyhedral protein is arrowed. Migration is from top to bottom.

Fig. 9.4 Polyacrylamide gel electrophoresis of CPV$_{A2}$ and NPV. The two outside channels (1, 10) contain standards; channels 2, 3, and 4 contain NPV polyhedral protein obtained after 5, 15, and 30 min alkali digestion; NPV polyhedra not treated with alkali is in 5; CPV$_{A2}$ polyhedra in 6, and different isolates of CPV$_{A2}$ virions in 7, 8, and 9. The CPV$_{A2}$ viral samples show low-mol.-wt. bands. Migration is from top to bottom.


a
b
b

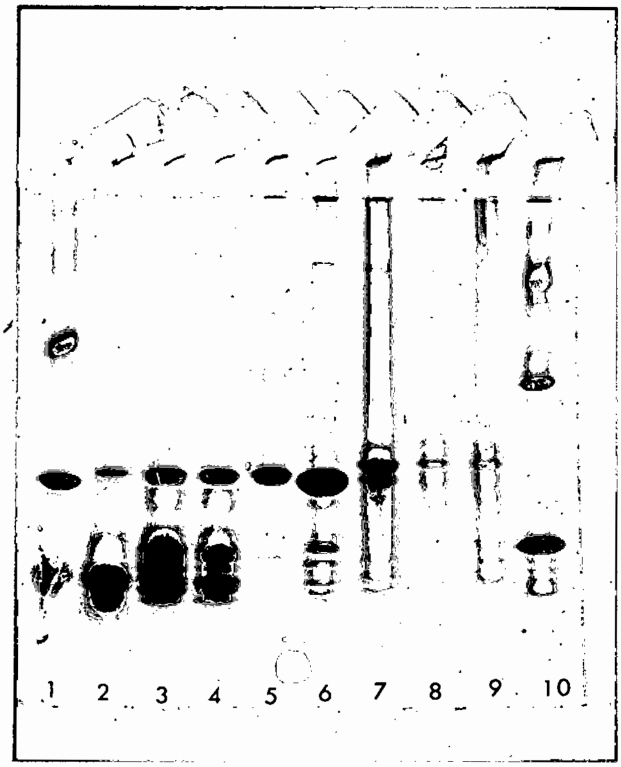

1
2
3
4
5
6
7
8
9
10

TABLE 9.3

POSSIBLE CORRESPONDENCE OF GENOME SEGMENT OF CPV$_{A2}$ TO THE SIZE OF VIRAL AND POLYHEDRAL PROTEINS

CPV$_{A2}$ genome segment number	Molecular weight				
	Nucleic acid (x 10^{-6})	Estimated ss RNA transcript (x 10^{-6})	Estimated* gene product (x 10^{-3})	Observed viral protein (x 10^{-3})	Observed polyhedral protein (x 10^{-3})
1	2.55	1.28	142	{163 139	145
2	2.15	1.08	120	125	
3	1.90	0.95	105		95
4	1.58	0.79	87		
5	1.10	0.55	61		
6	1.03	0.52	57		51
7	0.71	0.36	40	37	
8	0.58	0.29	32	32	
9	0.47	0.24	26	29	{29 25
10	0.40	0.20	22		

* = According to Lewandowski and Traynor, 1972.

At least ten bands were observed in the case of NPV polyhedra, ranging in mol. wt. from approximately 14 000 to 90 000. The major protein of NPV polyhedra had a mol. wt. of approximately 28 800. Polyhedral protein obtained after alkali digestion of polyhedra for 5, 15, or 30 min, revealed the presence of the major polyhedral protein with a mol. wt. of 27 900 and a marked increase in the quantities of low mol. wt. bands (Fig. 9.4). These bands were present in trace amounts in undigested polyhedra and ranged in mol. wt. from 11 500 to 25 800. Their mol. wts. were 11 500; 12 700; 14 200; 15 300; 16 600; 23 400 and 25 800.

The major band of both CPV and NPV polyhedra was shown to be a glycoprotein by staining the gels with Schiff's reagent (Fig. 9.5).

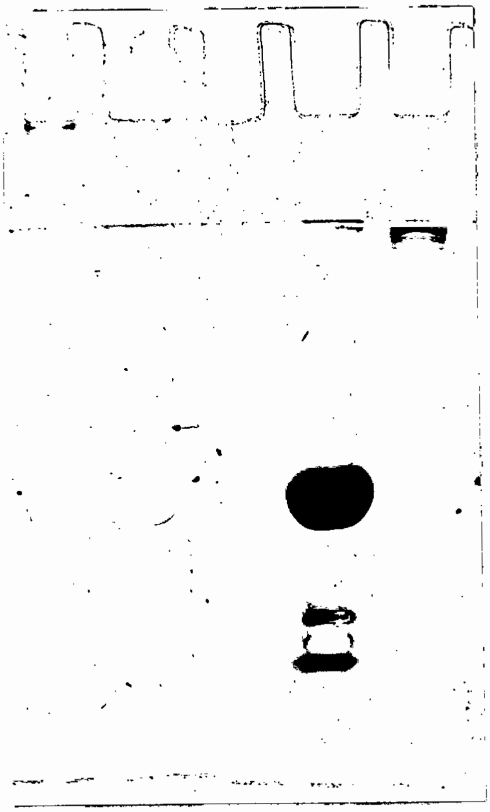


Fig. 9.5 Polyacrylamide gel electrophoresis of CPV_{A2} polyhedra on 12% gel. The left-hand side of the gel was stained with Schiff's reagent for glycoprotein while the right-hand side was stained with Coomassie blue.

9.3 DISCUSSION

The polyhedra of both CPV and NPV contain virus particles embedded in a protein matrix. These particles only comprise 2-5% of the inclusions. The major protein component in samples of polyhedra will therefore be of polyhedral origin.

Molecular weights in the region of 26 000 - 30 000 have been determined for the major proteins of several CPV polyhedra (Lewandowski and Traynor, 1972; Payne and Kalmakoff, 1974; Payne and Rivers, 1976). This agrees with the value of 28 700 found in this study for the main polyhedral protein of CPV. Viral proteins for CPV of *H. armigera* were found to range in mol. wt. from approximately 17 000 to 163 000, whereas Payne and Rivers (1976) determined a range of 26 000 - 149 000 for thirty-three CPV isolates. The lower mol. wt. bands detected in CPV of *H. armigera* could be breakdown products, persistent contaminants, or genuine viral proteins. Summers (1975) observed that the pH during solubilization was critical; at a pH above neutrality, degradation to low mol. wt. proteins occurred.

Payne and Kalmakoff (1974) determined mol. wts. of viral and polyhedral polypeptides of CPV of *B. mori* and calculated the theoretical size of protein products of translation from mol. wts. of the nucleic acid genome segments. The size of the CPV structural proteins was found to be close to the sizes of the theoretical primary gene products from certain RNA segments, as had been shown by Lewandowski and Traynor (1972). On the same basis, a possible relationship is indicated between the coding capacity of the separate RNA segments of CPV of *H. armigera* and the mol. wts. of some of the structural proteins (Table 9.3). It is possible that genome segment number 8 codes for the major viral protein with a mol. wt. of 32 400 and that the proteins with mol. wts. 163 000 and 139 000, and 37 000 are coded by gene segment numbers 1 and 7. Similarly, segments 1, 3, 6, and 9 may code for the polyhedral proteins with mol. wts. of 145 000, 95 000, 50 500, and 28 700 respectively. This is assuming that these high mol. wt. proteins do not represent aggregates.

Although Payne and Rivers (1976) could relate the size of most CPV structural proteins to those of the theoretical primary gene products from certain RNA segments, there were several exceptions which they postulated were due to post-translational cleavage of primary gene products. This may apply to the proteins of CPV of *H. armigera* that do not correspond in mol. wt. to theoretical primary gene products.

The major polyhedral protein of CPV of *H. armigera* was shown to contain carbohydrate. This result is similar to those of Payne (1971) and Payne and Kalmakoff (1974), who showed that the major polyhedral protein of the CPVs of *N. io* and *B. mori* contained carbohydrate.

In the present study, a mol. wt. of 28 800 was calculated for the major protein of NPV polyhedra of *H. armigera*; a protein of 25 400 present in lesser amounts and minor components of 17 000, 14 000, and 12 000 were also observed. Samples of polyhedral protein obtained after alkaline digestion of polyhedra showed a marked increase in the amounts of low mol. wt. bands. Similarly, Kozlov *et al.* (1975) found an approximate mol. wt. of 28 000 for polyhedral protein of NPV of *B. mori* and *Galleria mellonella*; after alkaline digestion of the polyhedra, they found a major component of 11 000 to 14 000 and a minor one of 23 000, which they suggested was as a result of cleavage of the major component of 28 000 by an alkaline protease. The minimal subunit of *B. mori* NPV polyhedral protein had a mol. wt. of 11 000 (Kozlov *et al.*, 1969). It appears that the polyhedral protein of NPV from different hosts is remarkably similar in mol. wt. The polyhedral proteins of NPV of *Porthetria dispar* were also found to be 29 000, 18 000, and 11 000 (Padhi *et al.*, 1974) which are similar both to those determined for NPV of *H. armigera* in the present study and to values found by Kozlov *et al.* (1975).

In the present study similar mol. wts. were obtained for the major polyhedral proteins of both NPV and CPV isolated from *H. armigera*. This is compatible with an earlier study by Kawase (1964) who showed that the amino acid patterns of polyhedral proteins of the NPV and CPVs of *B. mori* were similar. In contrast, Payne (1971), found that the polyhedral proteins of NPV and CPV isolated from the same host, *N. io*, were different in size; he postulated that the protein was therefore virus- and not host-specific. In the case of occluded viruses from *H. armigera*, the implication is that the polyhedral protein was host- rather than virus-specific.

CHAPTER 10

CONCLUSIONS

This thesis describes a study of the viruses extracted from larvae and pupae of *H. armigera*. Three occluded virus particles were found, viz. NPV, CPV, and GV. CPV was present both in the free and occluded state.

Throughout this study emphasis was placed on the properties specific for CPV, since this particular virus had not previously been examined in any detail. An essential step was the separation of CPV from mixtures of NPV and CPV in which it was present in only small quantities.

The size similarity of NPV and CPV polyhedra at the time of larval death caused by NPV, made their separation by differential centrifugation difficult. Released virions were separated by a combination of differential centrifugation, zone electrophoresis, and antibody complexing. After the initial isolation of CPV, the viruses were propagated in laboratory-reared larvae and each virus type purified. The CPV particles were found to be remarkably stable even after intensive purification, in contrast to some previous reports in the literature (Smith, 1967).

Contamination by unwanted viruses was considerably reduced by surface-sterilization of the eggs and pupae and strict adherence to sterility techniques. However, the findings in this study did not resolve the question as to whether latent virus is transmitted via the egg surface or transovarially.

CPV isolated from *H. armigera*, viz. CPV_{A1}, CPV_{A2} and CPV_{B} were morphologically indistinguishable from each other. The physical properties of the virions were similar, but the viruses could be distinguished by their nucleic acid profiles on polyacrylamide gels. This method appears to be invaluable for identifying different strains of the viruses.

The present study showed the virions of GV and NPV to resemble each other morphologically and showed them to be also serologically related. CPV differed in all respects investigated, from NPV and GV, except in shape and size of inclusion body, which was similar to that of NPV.

A comparison of the symptomatology of all three virus infections was undertaken. NPV caused the greatest mortality in the shortest time which underlines its potentiality as a pest control agent for *H. armigera*. Larvae also died from GV, but the larval stage, and therefore the feeding time, was prolonged, indicating that it is not as suitable as NPV for control of *H. armigera* infestations in the field. CPV did not kill the larvae but tended to modify the lethal effects of NPV in mixed infections as indicated by slight reduction in total mortality.

CPV is widely distributed throughout nature; it is not as host specific as previously thought, and the host can survive infection despite the presence of large numbers of viral particles. In general, the group of baculoviruses, which includes NPV, kill the host in a relatively short time.

The shape, size, and structure of the virus particle was revealed by negative staining and electron microscopy. The technique of immune electron microscopy confirmed the interaction of antigen and antibody, and it was possible to observe the linking of antibody to antigen in complexes. Advantages of this technique are its specificity and the low concentration of material required. The large particle size and slow diffusion rate of CPV of *H. armigera* in agar gel, precluded the use of double diffusion of whole particles. However, the particles did diffuse in low agar concentrations as in the gel precipitin technique. The technique of immune osmophoresis was sensitive in the detection and identification of low concentrations of reagents. Analysis of the serological relationships of the viruses was restricted to reactions only with surface antigens. The cross-reaction between *B. mori* CPV and the CPV of *H. armigera* was of interest since this virus is the first one to show a definite relationship with *B. mori* CPV, despite different band patterns of the nucleic acids on polyacrylamide gels.

With light microscopy, all stages of development of CPV polyhedra were seen in the columnar epithelial cells. Few polyhedra were observed early in infection and their development time was longer than NPV polyhedra. In larvae infected only with CPV, columnar epithelial cells became filled with polyhedra. Electron microscopy of thin sections revealed the production of virus particles both in the virogenic stroma and crystallogenic matrix. In addition, polyhedral formation and the incorporation of viruses into polyhedra could be observed.

Electron microscopy also indicated the possible presence of a membrane enclosing the larger mature CPV polyhedra, but in smaller polyhedra it was not distinct. CPV polyhedra were able to be digested by trypsin, which is in contrast with previous studies on NPV polyhedra (shown to be membrane-enclosed) which were only digested by a combination of trypsin and DNase. There have been no reports in the literature on enzyme digestion studies of CPV inclusion bodies.

Histological studies on NPV-infected larvae indicated a rapid progression of the disease. Most larval tissues were infected, with the exception of the midgut. Examination of thin sections of NPV-infected fat body cells, indicated that viral replication occurs in regularly assembled groups of virions. In cross-section, different numbers of nucleocapsids were seen grouped together in packets. However, when NPV was released from polyhedra by digestion of the inclusion body, single virions enclosed in viral membranes were observed. It is unclear whether both multiply and singly-enveloped virions occur in *H. armigera.*

Light microscopic examination of GV-infected fat body cells revealed the darkly staining network typical of GV infection. No infection of the midgut was apparent. In thin sections, different stages of virus production and encapsulation of particles could be seen in fat body cells in the electron microscope. Larvae infected with GV showed proliferation of fat body cells in conjunction with an extended larval period. From histological studies, the proliferation appears to be hyperplastic rather than neoplastic.

It would be interesting to determine whether the delicate hormonal balance of juvenile hormone (JH) and ecdysone has been disturbed by GV replication in the fat body cells, resulting in delay of the pupal moult. The production and accumulation of food reserves within fat body and other tissues in uninfected larvae may serve as a stimulus for the pupal moult. Depletion of these stores because of viral infection, may be a contributory factor in the extension of the larval stage. It has not been determined to date whether the number of larval instars differs in GV-infected larvae as compared to uninfected larvae.

The biophysical properties of the CPV particles of *H. armigera* were similar to other members of the Reoviridae. The CPV particle is icosahedral, and approximately 59 nm in diameter with surface projections measuring up to 27 nm in length. Physical parameters of the CPV virus which were determined, include a buoyant density of 1.44 g cm^{-3} by isopycnic centrifugation in CsCl gradients. This hydrated density value was similar to $1/\bar{v} = 1.43$ g cm^{-3}, the unhydrated density value.

To determine the partial specific volume of CPV, it was necessary, because of the low concentrations of CPV available, to determine the sedimentation rates of the virus in H_2O and D_2O in an analytical centrifuge fitted with ultra-violet optics. A partial specific volume of 0.69 ± 0.02 g ml^{-1} was obtained. This value is necessary in order to calculate other physical constants of the virus. No other values for $\bar{v}$ of CPVs are available for comparison.

A diffusion coefficient of 0.73×10^{-7} cm^2 s^{-1} was determined, using a modified gel diffusion technique, but this value is considered slightly high. The sedimentation coefficient of CPV virus was calculated as 399 ± 10 S corrected to standard condition at 20^0C with pure water as solvent and extrapolated to infinite dilution. This value falls within the range of *s* determined for other CPVs (Table 1.1.).

The molecular weight of CPV determined by combination of *s* and *D* (43.4×10^6) is considered low, possibly because of the high value of *D*. The particle counting technique used to determine molecular weight yielded a value of 51.5×10^6, which may be considered high. These findings are similar to those described by Marais (1970) for the particle weight of the β particle of *N. cytherea cytherea* in which a low value was obtained using *s* and *D*, and higher values found using the particle counting technique.

The equilibrium centrifugation method was thought to be the most accurate method to determine the CPV particle weight. For the large particles of this size, a low speed was necessary to prevent the particles sedimenting, but at this speed, precession became a problem. This was overcome by using an antiprecession device to steady the axis of rotation. Fringe measurements were thus possible, and a value of 47.25×10^6 was obtained. An average value of 47.4×10^6 was calculated from the three methods, which is close to that derived by equilibrium centrifugation. This value was obtained for the laboratory-passaged CPV_{A2}, which lacks the high-molecular weight RNA segment present in CPV_{A1}. To date, insufficient material has been obtained from field collections of larvae to determine the molecular weight of CPV_{A1}.

Because of different experimental approaches, reported values for the particle weight of CPV of *B. mori* range from $28\text{-}97 \times 10^6$ with an intermediate value of 54×10^6.

The nucleic acid of CPV was shown to contain ribose, whereas GV and NPV contained deoxyribose; buoyant density studies confirmed that CPV contained RNA, while GV and NPV contained DNA. Thermal denaturation studies demonstrated the double-stranded (ds) nature of all three viruses. The ds RNA of CPV separated into ten segments on PAGE, whereas the DNA of GV and NPV migrated as one slightly diffuse band.

Molecular weight values for the genome segments of CPV were determined by comparative PAGE whereas for NPV and GV, restriction endonuclease digestion would have been required to separate the genome components on PAGE.

The plot of log molecular weight versus electrophoretic mobility for the ds RNA deviates markedly from linearity at high molecular weight values. Use of a linear plot, in conjunction with reovirus RNA as standard, would result in marked underestimations of molecular weight values of the larger segments. In this study, the plot was calibrated with a single species of known molecular weight, an average for the 'M' segments of reovirus 3. The non-linear nature of the relationship was also confirmed by using the replicative form of foot and mouth disease virus, which is a ds species of very high molecular weight.

When comparative gel electrophoresis is used for molecular weight determination of ds RNA, a calibrated curve of the type utilized in this study, should be constructed in each laboratory with a suitable set of standards. CPV_{A2} RNA is an excellent marker because all the genome segments are well separated on PAGE unlike other members of Reoviridae and cover a larger molecular weight range than reovirus. For a complete set of markers, CPV_{A2}, in conjunction with ø6, and perhaps Hm 9 - virus-like particles of *Helminthosporium maydis*, would cover a wide range of molecular weight values.

An application of the method was demonstrated when CPV_{A2} was used as a standard in determining the molecular weight of CPV_B and CPV of *N. cytherea cytherea* by co-electrophoresis of the viral nucleic acids with that of CPV_{A2}. From the relative electrophoretic mobilities, the molecular weight of these CPVs could be determined.

An unusual phenomenon was observed in this study. This was the finding of a CPV whose genome pattern on PAGE was altered on passage in laboratory-reared host insects. A high-molecular weight segment disappeared and was replaced by a fast-moving low-molecular weight segment. This may have resulted from a mutation because of altered environmental conditions, or may be a result of the stimulation of a latent virus. The two genome profiles were identical in every respect with the exception of one major genome segment, which was absent in the passaged virus but was replaced by the presence of a low-molecular weight segment.

The major protein component of the polyhedra is a glycoprotein with a molecular weight of approximately 28 000. This was found in the polyhedra of CPV_{A2}, and NPV of *H. armigera* and the similarity in size may indicate that polyhedral protein is host-coded. The major protein of the CPV_{A2} virion had a molecular weight of approximately 32 400. Some of the polyhedral and viral proteins corresponded in molecular weight with those of the theoretical primary gene products of individual RNA segments.

Attempts made to grow NPV of *H. armigera* in microorganisms, viz. *B. subtilis* and *S. cerevisae*, were unsuccessful. Although the virus survived in the microorganism culture medium and produced infection in host larvae, no evidence of increased replication in the microorganisms was noted. Since the work was completed, it has been reported (Summers, 1975) that virions obtained by alkaline digestion of inclusion bodies are not infective, or have reduced ability to infect tissue culture, compared with the infectivity of free virus particles. This may equally apply to the system used to infect microorganisms in both the present study, and in the study described by Wells and Heimpel (1970). It would be of interest to test this system using virus particles occurring free in the haemolymph.

Although baculoviruses were initially thought to be restricted to insects, the Reoviridae, which include CPV, infect a variety of hosts that include man, insects and plants. However, recent reports have described nonoccluded particles resembling baculoviruses in red mites, *Panonychus ulmi* (Bird, 1967) and beetles, *Oryctes rhinoceros* (Payne, 1974). Recent studies indicate that a particle similar to NPV, occurs also in a crustacean, the pink shrimp, *Penaeus duorarum* (Couch, 1974). A further extension of the host range is indicated by the finding of large numbers of a similar particle in hyphae of the insect-parasitic fungus, *Strongwellsea magna* (Federici and Humber, 1977).

The apparent widespread distribution of particles resembling insect viruses indicate the need for increased study of this important group of viruses.

APPENDIX 1

ATTEMPTS AT GROWING INSECT VIRUSES IN MICROORGANISMS

Several attempts to grow the nuclear polyhedrosis virus of *Heliothis armigera* in microorganisms as suggested by Wells were unsuccessful.

MATERIALS AND METHODS

Virus inoculum:

Larvae of *Heliothis armigera* were used as a source of the nuclear polyhedrosis virus (NPV). Polyhedra were purified by differential centrifugation and treatment with fluorocarbon (Bergold, 1959). Virions were liberated by digestion with 0.5 to 1.0 ml of an alkaline solution of *p*H 10.4 (0.02 M Na_2CO_3 : 0.05 M NaCl, 1 : 1) added to 1-2 × 10^6 polyhedra/ml and incubated for 45 min at room temperature. Undissolved material and partially digested polyhedral protein were removed by low speed centrifugation. The supernatant fluid was diluted with 0.06 M phosphate buffer, *p*H 7.0, and used as a source of virus.

Host cells:

Saccharomyces cerevisiae and *Bacillus subtilis* were used. *S. cerevisiae* samples taken from agar slopes were inoculated into sterilized medium consisting of 0.5% (*m*/v) sodium chloride, 0.25% (*m*/v) disodium phosphate, 2% (*m*/v) tryptose casein hydrolysate, and 0.2% (*m*/v) dextrose in distilled water (Wells, 1970). *B. subtilis* was grown in the minimal medium used by Spizizen (1958) which consists of 0.2% (*m*/v) ammonium sulfate, 1.4% (*m*/v) dipotassium phosphate, 0.6% (*m*/v) monopotassium phosphate, 0.1% (*m*/v) sodium citrate ($2H_2O$), 0.02% (*m*/v) magnesium sulfate ($7H_2O$), and 0.5% (*m*/v) glucose supplemented with 0.01% (*m*/v) Difco yeast extract in distilled water.

Experimental procedure:

The microorganisms were passaged several times and at the peak of their logarithmic growth phase harvested by low speed centrifugation. The pellet of cells was suspended in sterile distilled water and recentrifuged, and the final pellet was suspended in the "transformation medium" of Wells (1970), which consisted of 15% (*m*/v) sucrose, 2% (*m*/v) magnesium sulfate, 0.2% (*m*/v) sodium acetate, and 0.01% (*m*/v) tryptone in distilled water. The cells were left in the medium for 1 h with light agitation and then mixed with a small volume of prepared virus suspension in buffer. The cultures were incubated with light agitation for the first 2 h at 28°C for *S. cerevisiae* and 32°C for *B. subtilis*. Control cultures of the microorganisms without admixture with virus were treated in the same way.

An alternative method of growing *B. subtilis* in differing media was used (Romig, 1962). The *B. subtilis* suspension was calibrated to achieve an 0.3 OD unit at 600 nm and incubated in changes of medium prior to incubation of the culture in the "transformation medium" and addition of the prepared virus suspension.

Microscopy:

Light microscopy was used to examine wet mounts and dried stained smears (Smirnoff, 1961) for the presence of polyhedra. Negatively stained bacterial suspensions sedimented at 7000*g* in the Spinco 40 rotor for 10 min were examined for polyhedra by electron microscopy. The supernatant fluid was centrifuged at 30 000*g* in the same rotor for 30 min and the pellet examined for the presence of free virus particles. Pellets of bacteria obtained by centrifugation were incorporated in 1% agar, fixed in 1% osmium tetroxide buffered at *p*H 7.0, dehydrated, and embedded for thin sectioning. Sections were stained with lead citrate and uranyl acetate (Reynolds, 1963) and examined by electron microscopy.

Infectivity:

Infectivity experiments were conducted on *H. armigera* larvae reared from surface-sterilized eggs on a semi-synthetic growth medium (Bot, 1966). The larvae were incubated at 30°C for five days then placed on infected growth media and kept under observation at 26°C for six to eight days. Control uninfected larvae were kept under the same conditions.

RESULTS

Cultures:

Suspensions of virus and microorganisms were mixed and incubated in the following manner:

(1) NPV[1] with *B. subtilis*;

(2) NPV suspended in the "transformation medium" without *B. subtilis*;

(3) NPV suspended in bacterial culture media after removal of the organisms by filtration;

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[1] Virions derived from digestion of 10^6 to 10^7 polyhedra/ml.

(4) *B. subtilis* alone;

(5) NPV with *B. subtilis* was subcultured, after the conclusion of one experiment, into a fresh culture of *B. subtilis*;

(6) NPV with *S. cerevisiae* and the mixture compared with a pure culture of *S. cerevisiae*.

The cultures were incubated as described under Materials and Methods and aliquots were taken at intervals of four to eight days. Following centrifugation, pellets of *B. subtilis* were repeatedly frozen and thawed using solid CO_2 in acetone as the freezing mixture in order to disrupt the bacteria and liberate any virions present. Attempts to disrupt the cell wall of *S. cerevisiae* after conclusion of the experiment by ultrasonication, freezing and thawing, and detergent treatment were unsuccessful but approximately 10% of the cells could be disrupted using a pressure disintegrator (Polson, 1973).

Microscopy:

Polyhedra were not detected in any of the preparations examined by light or electron microscopy. Examination of *B. subtilis*-NPV cultures revealed empty nucleocapsids and full virions lying only outside the cells. No virus was detected within the cells. A similar situation was observed in the *S. cerevisiae*-NPV cultures. More empty NPV nucleocapsids were seen in the frozen and thawed material (Fig. A1) but more full NPV virions were observed in virus suspensions incubated in the "transformation medium" than in virus suspensions incubated in the bacterial extracts. No difference in the number of empty nucleocapsids was observed in material derived from the different incubation methods (Spizizen, 1958; Romig, 1962). No intracellular virus particles could be detected in any of the thin sections of yeast or bacterial cells, although some virus

Fig. A1 Empty NPV nucleocapsids seen after freezing and thawing *B. subtilis* previously inoculated with NPV (x 75 000).

Fig. A2 Thin section of *B. subtilis* NPV preparation. Virus particles are seen lying outside the bacterial cell. No particles are visible within the cell (x 78 000).

particles could be seen outside both the bacterial and yeast cells (Fig. A2).

Infectivity:

Infectivity experiments were conducted on *B. subtilis*-NPV, NPV in the "transformation medium", and the NPV suspension used for seeding *B. subtilis* culture and "transformation media". The same volume of virus inoculum had been used in each case. In addition *B. subtilis* suspension in the absence of virus was also tested. Identical incubation times and temperatures were used. At the conclusion of the experiment, the media containing the different suspensions were concentrated by pervaporation and made up to a fixed standard volume to make them strictly comparable.

Prior to use the samples were subjected to two cycles of freezing and thawing. Serial ten-fold dilutions of the suspensions were prepared and equal volumes of the different suspensions, including the undiluted material, were added to the semisynthetic growth medium. Eight larvae were used for each dilution. Infectivity experiments were also conducted on *B. subtilis*-NPV subcultured material and *S. cerevisiae*-NPV.

The results of the *B. subtilis* infectivity experiments are represented in Fig. A3. No significant difference could be observed between the various groups studied. Larvae infected with subcultured *B. subtilis*-NPV material did not show increased mortality as compared with control uninfected larvae. Neither was there a significant increase of mortality of larvae infected with *S. cerevisiae*-NPV.

From the infectivity experiments, larvae which died were examined, and large numbers of NPV were revealed by electron microscopy (Fig. A4). No virus was recovered from larvae infected with the material subcultured in *B. subtilis* or from the control uninfected larvae (spontaneous mortality rate 0.5%).

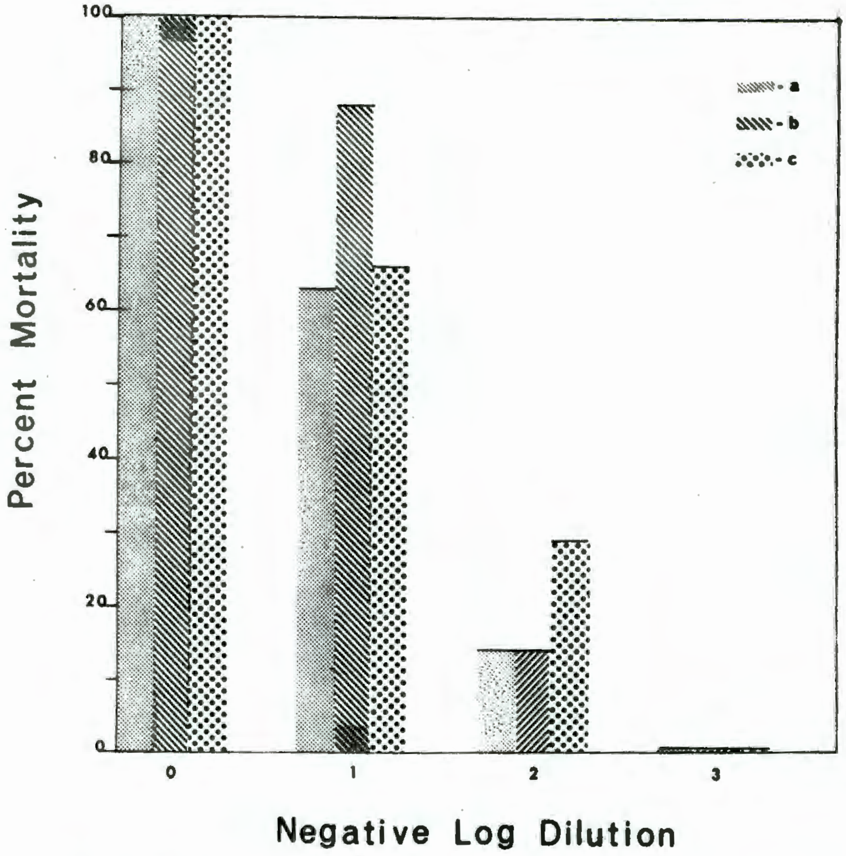

Fig. A3 Results of infectivity experiments. Percentage mortality as a function of serial dilutions of different virus cultures represented as histograms: (a) NPV inoculum; (b) NPV in "transformation medium"; (c) NPV-*Bacillus subtilis*. The histogram at negative log dilution 3 represents zero mortality.

Fig. A4 Virus recovered from *H. armigera* larvae fed *B. subtilis* - NPV suspension. Polyhedra had been digested by alkali (x 100 000).

DISCUSSION

Attempts at growing NPV of *H. armigera* in the microorganisms *S. cerevisiae* and *B. subtilis* were unsuccessful despite several variations in the culture methods. The morphological appearance of bacteria and yeast cells in both the control and "infected" cultures was not significantly different.

Wells (1970) used the term "transformation medium", but in a subsequent paper (Wells and Heimpel, 1970), the percentage of cells which were indeed transformed was not stated. Using the same conditions which Wells used, a small number of cells were found which showed dissociation of the protoplast from the cell wall, i.e. the appearance described by Wells of cells in the "wall altering" (Wells and Heimpel, 1970) or "transformation" *media* (Wells, 1970). The bacterial cells were broken with freezing and thawing but the cell wall of *S. cerevisiae* proved resistant to most methods of disruption.

On no occasion was any direct evidence obtained of virus replication or of intracellular virus. This is supported by the following observations:

(1) There was an increase in the number of extracellular empty virions in the medium containing microorganisms as compared with the medium alone, but since this occurred to the same extent in bacterial extracts this suggested degradation, probably by bacterial enzyme action, rather than entry of DNA into the cell with release of the protein coat.

(2) Electron microscopical examination of virus in bacterial "infected" cultures and in control medium without microorganisms showed a similar number of complete virions. The virus particles present at the end of incubation with microorganisms were not adsorbed to the cell surface nor intracellularly situated.

(3) Mortality in larvae infected with bacterially cultured NPV was no higher than larvae infected with NPV cultured in the absence of bacteria. No larvae succumbed to NPV disease after infection with *B. subtilis*-NPV subcultured material.

These investigations therefore failed to confirm Wells' (1970) observations that insect viruses replicate in living microorganisms, but are in agreement with Dr. Tinsley (pers. comm.) who was unable to demonstrate replication of insect viruses in yeast cells or in yeast protoplasts.

APPENDIX 2

CONDITIONS OF CENTRIFUGATION

In this thesis conditions of centrifugation are described in units of gravity (*g*) together with an indication of the rotors used. This is necessary since the specification of *g* alone does not allow one to reproduce exactly the conditions of centrifugation. The efficiency of sedimentation depends, for instance, on the angle of the tube in the rotor, and if the geometry of the rotor is unknown, information in terms of *g* is of little use.

Some of the specifications of the rotors used are listed in Table A2. Tables published by Spinco Division of Beckman Instruments give further information. A Spinco Model L ultracentrifuge was used throughout the study.

TABLE A2

SPECIFICATION OF ROTORS USED

	Swing-bucket rotors			Anglehead
	SW 39L	SW 50.1	SW 25.1	Type 40
Maximum speed	40 000	50 000	25 000	40 000
Centrifugal force at maximum speed (R_{ave})	125 000*g*	234 000*g*	64 700*g*	105 400*g*
Tube angle	90°	90°	90°	-
R_{max} (mm)	98	107.3	129.0	81
R_{min} (mm)	44	59.7	56.0	38

APPENDIX 3

ABBREVIATIONS AND SYMBOLS

$A_{260}nm$: Absorbance at 260 nm

BTV : Blue tongue virus

CPV : Cytoplasmic polyhedrosis virus

D : Diffusion coefficient ($cm^2\ s^{-1}$)

DNA : Deoxyribonucleic acid

DNase : Deoxyribonuclease

ds : Double-stranded

FDV : Fiji disease virus

FMDV : Foot and mouth disease virus

g : Acceleration due to gravity ($980\ cm\ s^{-2}$)

GV : Granulosis virus

mol. wt. : Molecular weight

m : Mass

MRDV : Maize rough dwarf virus

NPV : Nuclear polyhedrosis virus

η : Viscosity (poises)

PAGE : Polyacrylamide gel electrophoresis

PB : Phosphate buffer, 0.06 M, pH 7.0

PIB : Polyhedral inclusion bodies

PBS : Phosphate buffered saline

PEG : Polyethylene glycol

PPRI : Plant Protection Research Institute

r : Radius (cm)

R : Gas Constant ($8.314 \times 10^7 erg/^0C/mole$)

n : Refractive index

R_{ϕ} : Ratio of the distance migrated by the virus in the zone electrophoresis column, to the distance migrated by the phenol red indicator.

RDV : Rice dwarf virus

RF : Replicative form

RNA : Ribonucleic acid

RNase : Ribonuclease

s : Sedimentation coefficient (Svedberg unit = 10^{-13}s)

S : Svedberg unit
SDS : Sodium dodecyl sulphate
S.D. : Standard deviation
S.E.M. : Standard error of mean
SSC : Standard saline citrate
ss : Single-stranded
t : Time
T : Absolute temperature (^{0}K)
TEMED : N,N,N',N'-tetramethylethylenediamine
TMV : Tobacco mosaic virus
$\bar{\nu}$: Partial specific volume (ml g^{-1})
V : Volts
ρ : Density (g cm^{-3})
ω : Angular velocity (radians s^{-1})
WTV : Wound tumour virus

APPENDIX 4

During the course of this study the following publications were prepared:

HARLEY, E.H., RUBINSTEIN, R., LOSMAN, M. and LUTTON, D. (1977) Molecular weights of the RNA genome segments of a cytoplasmic polyhedrosis virus determined by a new comparative approach. *Virology, 76,* 210-216.

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