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November 13, 1979
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ABSTRACT

In recent years, nuclear polyhedrosis viruses have been widely used as insecticides. These agents have been exhaustively tested against a number of animals and plants without any observed ill effects. However, in vitro studies suggest that nuclear polyhedrosis viruses are able to replicate in non-insect host systems without any outward signs of infection. To test this possibility, DNA-DNA hybridization experiments were conducted to detect the levels of viral DNA in African Green Monkey kidney cells following infection. The experiments demonstrated a rapid decline in the amount of viral DNA in the cells within 24 hours following infection. However, the decline did not result in the complete elimination of viral DNA from the cells and detectible levels remained after 48 hours. These results suggest that the viral genome is maintained at low levels in these cells. The possibility of viral persistence, replication and/or integration is discussed.
INTRODUCTION

The Control of Insects

The control of insects has been a matter for concern since man first began to grow his own food. This concern must certainly have grown with the world's population and the resulting demand for better and more efficient agricultural methods. Yet, it was not until recent years, with the advent of synthetic organic insecticides, that insect pest control was first realized.

Chemical Insect Control

The use of synthetic organic insecticides has been the basic insect control strategy since these materials first appeared in the late 1940's (for reviews see NAS 1975, Pimentel 1976). Insecticides, along with other agricultural chemicals, new plant varieties, and mechanization, have substantially increased crop production. USDA officials maintain that without these developments 70% of crops produced in the United States could not be successfully grown, and those which could would suffer additional losses with a resulting rise in food prices of 50 to 70% (Walker 1969, Shaw 1971).

The role of pesticides is not limited to agriculture. They are also important in the control of insect vectors which transmit such diseases as malaria, yellow fever, dengue fever, and filariasis (Durham 1969, Wright, Fritz and Haworth 1972, NAS 1973).
However, there are problems associated with the use of chemical pesticides (Luck, van der Bosch and Garcia 1977). Residual toxicity is one outcome of their use which has received widespread attention (Niering 1968, Miller and Berg 1969, Hulbert 1975). The development of resistance in the target insects and the "insecticide treadmill" are problems which have been reviewed by Georghiou (1972) and Georghiou and Taylor (1977).

Biological Insect Control

An alternative to the unilateral use of chemical pesticides involves the commercial development of insect-specific biological agents such as viruses (WHO/FAO 1973). Luck and co-workers (1977) suggest that the best approach to controlling insect pests is to integrate natural and artificial mortality factors into an economically viable pest management program. This approach makes use of natural insect control agents as one "tactic" in an overall control "strategy."

Awareness of the potential of viruses as control agents for insect pests dates from the 1930's. At that time, the European spruce sawfly, Gilpinia hercyniae, which was causing catastrophic damage to spruce forests in Canada, began to decline in numbers. Within a few years these insects were no longer economically important pests. The reason was found to be infection by a virus (Balch 1939, Balch and Bird 1944).

Viruses have now been isolated from more than 400 species of insects and mites. Most of these are from insects in the order Lepidoptera, though viruses have also been identified in species of
the orders Hymenoptera, Ephemeroptera, Orthoptera, Hemiptera, Neuroptera, Trichoptera, Coleoptera, and Diptera (for reviews see WHO/FAO 1973, David, 1975, Tinsley and Harrap 1978). Insect and mite viruses infect some of the most important agricultural and forest pests in the world, including grasshoppers, the codling moth, many species of cutworm, armyworm, several species of sawfly, defoliating caterpillars, and the citrus red mite (WHO/FAO 1973).

In recent years several insect viruses have been used to combat agricultural and forest pests. In the United States, at least five viruses have been produced on a commercial or experimental scale for field use. All five are nuclear polyhedrosis viruses. Their primary hosts are species of the genera Heliothis, Neodiprion, Prodenia, Spodoptera, and Tricholoplusia (WHO/FAO 1973).

Insect Viruses

Classification

As with other viruses, the classification of insect viruses is based on the morphological and physical properties of the virions. Accordingly, insect viruses are distinguished by the type of nucleic acid they possess, their shape and dimensions, the presence or absence of an envelope, and their host range.

Reports from the International Committee on Nomenclature of Viruses (ICNV) (Wildy 1971, Fenner 1976) place some insect viruses in the genera Poxvirus, Parvovirus, Rhabdovirus, Enterovirus, and Icosahedral cytoplasmic deoxyribovirus (iridovirus). However, the most
common natural insect pathogens make up the family Baculoviridae. These are the viruses responsible for the nucleopolyhedrosis and granulosis diseases of many species of insects.

Baculoviruses

**Family Members.** The family Baculoviridae consists of the granulosis viruses (GV) and the nuclear polyhedrosis viruses (NPV) (Fenner 1976). The molecular biology of the baculoviruses has been recently reviewed by Summers (1978). GV and NPV are structurally similar. The enveloped nucleocapsids are typically 60 to 70 nm in diameter and 260 to 300 nm long (Harrap 1972b). They both contain DNA as their nucleic acid and replicate in the nuclei of infected cells.

**The Inclusion Body.** The characteristic structure associated with these viruses is a proteinic inclusion in which the rod-shaped enveloped nucleocapsids are randomly embedded. The NPV inclusions, called polyhedra, are large (1-15 microns) protein crystals and contain either enveloped single nucleocapsids (SNPV) (Figure 1) or multiple nucleocapsids common to a single envelope (MNPV) (Figure 2). The GV inclusions, called granules or capsules, are relatively small (0.2-0.4 microns) and contain only one enveloped nucleocapsid (Figure 3).

Mature inclusion bodies often show a peripheral electron dense layer in ultrathin sections. Dilute alkali solubilization of inclusion bodies can result in collapsed "baglike" ghosts of the polyhedra or granules. Such structures have been called polyhedron membranes.
Figure 1. Polyhedra with enclosed singly-enveloped nuclear polyhedrosis virus (SNPV) x100,000 (Summers 1975)
Figure 2. Polyhedron with enclosed multiply-enveloped nuclear polyhedrosis virus (MNPV) x50,000 (Summers 1975)
Figure 3. Granule with enclosed granulosis virus x200,000 (Summers 1975)
Harrap (1972a, 1972c) has proposed that these membranes are synthesized de novo as the final step in inclusion body crystallization.

Polyhedra and granules are made up of protein subunits arranged in a cubic lattice structure (Bergold 1963a, 1963b). Summers and Egawa (1973) have named these protein subunits granulin for GV and polyhedrin for NPV. Alkali dissolution and treatment with sodium dodecyl sulfate (SDS) reduces the inclusions to their granulin or polyhedrin subunits. Electrophoresis in SDS-polyacrylamide gels reveals these molecules as 25,000 to 31,000 dalton proteins, depending on the virus studied (Summers and Egawa 1973, Kozlov et al. 1973, Padhi, Eikenberry and Chase 1975, Summers and Smith 1975, 1978, McCarthy and Liu 1976, Rohrmann 1977, Harrap, Payne and Robertson 1977, Brown, Bud and Kelly 1977). Chemical studies have shown the peptides to be phosphorylated and to contain a high concentration of hydrophobic amino acids (Summers and Egawa 1973).

Alkaline protease activity has been detected in the inclusions of some baculoviruses (Summers 1975, Summers and Smith 1975). It is not known whether this enzyme activity is associated with the polyhedrin granules subunits themselves or with another unidentified protein.

The primary structures of several polyhedrihs and granulins have been compared in peptide mapping experiments (Kozlov et al. 1975, Summers and Smith 1976, Cibulsky, Harper and Guadauskas 1977). This technique can discriminate single amino acid differences among major peptides. The "fingerprints" of these proteins were almost identical, but in all cases there were some differences, indicating regions of
non-similarity between molecules. These findings indicate that polyhedrins and granulins are viral-coded but group related proteins.

The Nucleocapsid. The fine structure of the baculovirus nucleocapsid has been examined by a number of workers (Kozlov and Alexeenko 1967, Himeno et al. 1968, Khosaka, Himeno and Onodera 1971, Hughes 1972, Harrap 1972b). It is composed of an outer envelope, a protein capsid, and an inner nucleoprotein complex.

The envelopes which surround these viruses have unit membrane trilaminar structure. They are added to the naked virion either by de novo synthesis in the nucleus or when the virus buds through the nuclear or cytoplasmic membrane of an infected cell (Volkman, Summers and Hsieh 1976). The rod-shaped capsid is composed of repeating protein subunits in a ring-like or helical conformation. Electron microscope observations suggest that one end structure of the nucleocapsid is involved in a nuclear pore interaction, possibly resulting in the release of viral DNA into the nucleus of the infected cell (Teakle 1969, Summers 1971, Stoltz and Vinson 1977). The viral genome is contained in the central core of the nucleocapsid in association with one or more core proteins comprising the nucleoprotein complex.

The Genome. The baculovirus genome is a supercoiled, double stranded DNA molecule. Estimates of molecular weight vary widely, depending on the virus studied and the technique used in the measurement. Most estimates have been derived by sedimentation in neutral sucrose gradients relative to bacteriophage DNA standards or by
renaturation kinetics (Summers and Anderson 1972a, 1972b, 1973, Harrap, Payne and Robertson 1977, Kelly 1977). The best size estimates were made by Burgess (1977) using electron microscopy to compare the contour lengths of baculovirus genomes to that of an internal standard. Using the replicative form of the bacteriophage F1 genome as the standard, Burgess measured the genomes of seven MPV and three GV. They were found to range in molecular weight from $58 \times 10^6$ to $94 \times 10^6$ daltons.


After ingestion inclusion bodies disassociate in the alkaline environment of the insect gut lumen. The nucleocapsid enters cells lining the gut by envelope fusion with the microvillar membrane. The nucleocapsid then traverses the cytoplasm to interact with a nuclear pore and release its DNA into the nucleus.

The latent period before the appearance of infectious virus varies from six to twenty-four hours. During this period viral "stroma" or replicative chromatin complexes appear in the nucleus and nucleocapsids are seen to assemble in association with these structures. As the nucleocapsids are completed, many escape the nucleus, enter the cytoplasm, and bud from the surface of the cell into the insect's hemocoel (blood). Summers (1971) proposed a cytoplasmic transport mechanism for this process. Virions released in this manner are referred to as
non-occluded virus (NOV), plasma membrane budded virus (PMB-NOV), or simply extracellular virus. Alternatively, many virions remain in the nucleus and obtain an envelope by de novo synthesis. The viral "stroma" are seen to condense forming the polyhedral or granular inclusion bodies which trap the enveloped virions. Large numbers of inclusions accumulate in the nucleus and finally disrupt the cell.

Virions released from the gut cells into the hemocoel carry the infection throughout the insect's body. Widespread tissue destruction follows, resulting in the syndrome which is characteristic of these agents. As the insect corpse degenerates the inclusion bodies are deposited on vegetation and soil, to be ingested by another insect.

Baculovirus replication in vitro simulates the secondary in vivo infection. Foci of infection develop where one or more NOV infect a cell and the resulting NOV progeny carry the infection to neighboring cells.

The factors which control the fate of the baculovirus nucleocapsids are not well understood. Release of extracellular virus and occlusion appear to be related, time and tissue dependent phenomena (Volkman, Summers and Hsieh 1976). Nucleocapsids which are assembled before the synthesis of some virus-specific protein, perhaps the polyhedrin-granulin needed for occlusion, are able to escape the nucleus. Those assembled after the synthesis of this protein are trapped in the growing matrix and occluded.
Infectivity. As would be expected from the differences in their developmental pathways, occluded and non-occluded viruses differ in their infective properties. Occluded virus is only infectious when released in the insect gut. Artificially alkali-released virus is not infectious in tissue culture, when injected into insect hemocoel, or even when fed to insects (Volkman et al. 1976, Volkman and Summers 1977, Shapiro and Ignoffo 1969). Summers (1975) suggested that inclusion dissolution in vivo, by way of the virus' alkaline protease, alters the viral membrane in a way which has not been duplicated in the laboratory; thus, the lack of infectivity in the alkali-released viral preparations. Non-occluded virions have membranes which are morphologically dissimilar to those of occluded virions (Summers and Volkman 1976). NOV, whether produced in vivo or in vitro are non-infectious when fed to insects, but are highly infectious when injected into the hemocoel or inoculated onto cell cultures (Volkman and Summers 1977). Summers and Volkman (1976) compared in vivo and in vitro NOV and found them to be almost identical physically and morphologically. The results of these studies point to the importance of the viral membrane and its source of origin in determining the infectious properties of the virions.

Potential for Insect Control. Of all the insect viruses, baculoviruses offer the greatest potential as insect control agents (WHO/FAO 1973). The baculovirus inclusion bodies are easily produced, they can be stored for long periods without loss of infectivity, and they can be efficiently administered to crops in a water suspension. Another important property of these viruses is their limited host range. They
are apparently restricted to invertebrates and many members of the family appear to infect only a few species of insect. However, studies on baculovirus specificity are far from conclusive.

Heliothis NPV is currently formulated as a powder containing the inclusion bodies. This is suspended in water and sprayed on the crop in the same manner as chemical insecticides. This preparation has been used extensively on cotton and other crops. It has proven very effective on the target pest, the cotton bollworm, but does not appear to be harmful to parasites and predators of the target insect, to other animals, to plants, or to personnel.

Safety Testing of Insect Viruses for Use as Insecticides

Potential Hazards

While viruses may have a useful role to play in the control of insect pests, the hazards involved in the deliberate release of replicating virus into the ecosystem cannot be discounted lightly. Tinsley and Melnick (1974) suggest that there are three basic hazards which may arise from the use of these agents to control agricultural pests. First, the virus may infect insect hosts other than the target pest. Such infections could be fatal, or non-lethal and inapparent. Latent insect viruses could be activated resulting in a secondary epizootic with uncalculated consequences. Second, certain insect viruses may be able to directly infect invertebrates other than insects and perhaps even vertebrates. If such infections were inapparent, then these events would be almost impossible to detect. In evidence of this hazard, antibodies
reacting with insect viruses have been found in the sera of domestic and wild animals (Longworth et al. 1973) and in the sera of laboratory workers who handle these agents (WHO/FAO 1973). Finally, changes in the pathogenicity and specificity of pesticidal insect viruses could occur which would give rise to a wider spectrum of host involvement. This could happen through mutation, through recombination of genetic material, or by the acquisition of specific host material during passage in the alternate host.

Types of Tests Required

It has only been within the last two decades, largely as a result of industrial attempts to develop microbial insecticides, that safety testing of insect pathogens has been undertaken (for reviews see WHO/FAO 1973, Ignoffo 1973). Initially, only studies to evaluate the potential toxicity and/or pathogenicity of an agent were conducted. More extensive testing began after the agent was seriously considered as a potential microbial insecticide. The types of studies tentatively required by the Environmental Protection Agency (EPA) to evaluate the safety of future microbial insecticides, especially NPV and GV, are described below.

**Acute Toxicity-Pathogenicity.** Feeding, inhalation exposure, and topical toxicity are used to evaluate acute toxicity or pathogenicity of candidate materials in at least two species of laboratory animals. Dosages and exposure times are varied and median lethal dose ($LD_{50}$) and median lethal concentration ($LC_{50}$) are calculated where possible.
Microbial insecticides, known to be relatively non-toxic and non-pathogenic, are generally tested at 10 to 100 times the average field dose per acre.

Chronic Toxicity-Pathogenicity. Chronic or subacute studies are initiated after toxicity-pathogenicity has been established. The objective of these studies is to detect any long term effects that the agent may produce. The doses, selected from previous acute studies, are administered at levels anticipated to produce no effect, minimal measurable effect, and maximum effect. The agent is administered throughout the test period. The test material is administered in the diet or by inhalation, subcutaneous injection, or skin contact.

Primary Irritation to Skin or Eye. The skin and mucous membranes of the eye frequently are exposed to pesticides. Both tissues are used to evaluate an agent's potential to induce irritation. Rabbits are the animals of choice. Human subjects were tested to evaluate skin irritancy and potential sensitizing ability of the Heliothis NPV (Ignoffo 1968).

Teratogenicity. Teratology is the study of cause and development of malformations that occur prior to, at, or shortly after birth. In these studies the agent is repeatedly administered orally at subacute levels to pregnant animals. Rats are usually the animals of choice, though others are commonly used. Results are based on obvious malformations of the newborn.
Carcinogenicity. It is well established that certain RNA and DNA viruses, as well as other foreign substances, induce cell changes that ultimately cause malignancy. With viruses, this may occur without the formation of infectious virus particles. In these studies, tumor-igenicity is considered an index of potential carcinogenicity. Strains of rats, mice, or hamsters, specifically bred for susceptibility to carcinogens, are used. The tests are initiated when the animals are young and are continued for periods of 1 1/2 to 2 years.

In Vitro Replication. In safety testing, the most important question is whether a candidate insect virus can infect cells of vertebrates. If it is unable to infect such cells, only the allergic and toxic risks need to considered. The difficulties associated with testing for carcinogenicity and teratogenicity could be avoided if it were shown that neither virions nor their components were capable of infecting the cells of man and other important species.

Mutation and Selection for Pathogenicity. The probability that an insect pathogen will mutate or that strains will be selected that will infect other animals is virtually impossible to determine. The object of these studies is to try and induce genetic changes in the test agent, either by selective pressure or by exposure to mutagens, which alter its specificity and/or pathogenicity. Negative results do not rule out the possibility that these changes may occur in nature.
Invertebrate Specificity. Specificity of the insect pathogen for a single invertebrate species or for a group of closely related species is an important requirement. Infection of species other than the target insect could have devastating ecological consequences. It is for this reason that the test agent must be evaluated for infectivity in a wide variety of invertebrates. Microscopic, seriological, and physiological examinations for infection are made on the test animals. These animals include other insects, worms, spiders, and crustaceans.

Phytotoxicity-Pathogenicity. Adverse affects of the pathogen to plants must be evaluated. Usually, greenhouse-grown plants of species that represent different families of economically important plants are used.

Results of Studies on Baculoviruses

In Vivo. The Heliothis NPV has been the most extensively tested of all insect pathogenic viruses, not only against vertebrates but also against invertebrates and plants (see review by Ignoffo 1973). Initial studies proved that the virus was innocuous to white rats and guinea pigs after oral, intravenous, intradermal, interperitoneal, or intracerebral administration (Ignoffo and Heimpel 1965). From 1965 to 1970 at least 24 plants, 36 invertebrates, 4 birds, 7 fishes, and 6 mammals, including man, were exposed to heavy doses of Heliothis NPV without any adverse reactions. In some instances, doses greater than 1,000 times the field rate per acre were used. In no instance was toxicity or pathogenicity demonstrated in any organism other than Heliothis species.
Ignoffo (1973) lists an additional 37 baculoviruses which have been tested against more than 20 species of vertebrates without one report of harmful effects.

*In Vitro.* There are relatively few studies concerning the effects of baculoviruses on vertebrate cell cultures. Discussions of this subject can be found in reviews by Ignoffo (1973), McIntosh (1975), and Granados (1976).

Aizawa (1961) obtained some death in chicken embryos that were inoculated with *Bombix mori* NPV and found that the polyhedron morphology of the virus was changed in the process.

Himeno and co-workers (1967) reported that infectious DNA of *B. mori*-NPV induced the production of polyhedra in a small proportion of cultured human amnion FL cells. These polyhedra were isolated, digested with alkali, and injected into *B. mori* pupae. In three different experiments a total of 10 out of 14 inoculated pupae became diseased and developed typical NPV symptoms. Serological tests indicated that the polyhedra formed in FL cells shared common antigens with polyhedra produced in *B. mori* insects. However, these results have never been duplicated.

Ignoffo and Rafajko (1972) failed to show any deleterious effects of *Heliothis zea* NPV (released by alkali treatment of polyhedra) on four primate cell lines. Inoculated cultures did not agglutinate guinea pig erythrocytes or interfere with the development of Echo-virus II.
The results of McIntosh and Maramorosch (1973) support these findings. However, it was also demonstrated that virus could be recovered from inoculated cultures of primary human amnion (PHA), W138, and human leukocytes four weeks after inoculation. In addition, enhancement of transformation of PHA by simian virus 40 was demonstrated in one of three experiments with \textit{H. zea} NPV.

In further studies, PHA, hamster (BHK-21), human fibroblast (HF), frog haploid (ICR-2A), and viper (VSW) lines were inoculated with alkali released \textit{Trichoplusia ni} NPV. No multiplication of virus was observed after four weeks. However, virus was recovered from cell culture media. It was found that virus could be recovered from the BHK-21 cultures after three passages of this cell line over a four week period.

Similar results were obtained with \textit{Autographa californica} NPV in PHA, BHK-21, and ICR-2A cell lines. No virus multiplication was observed and a reduced amount of virus was found in the culture media after three to four weeks. Interestingly, it was seen that \textit{A. californica} NPV multiplied in the \textit{T. ni} cell line (TN-368) at 37°C. This insect line does not multiply at 37°C but can be maintained at this temperature for at least four days (McIntosh and Rechtoris 1974). This finding indicates that the temperature of 37°C is not restrictive to the virus (which normally replicates at 28°C) nor is cell division necessary for viral multiplication.

In a recent study, McIntosh and Shamy (1975) observed the replication of \textit{A. californica} NPV in a viper (VSW) cell line. Virus labelled with tritiated thymidine was used to infect the cells.
Seventy-two hours after the infection autoradiograms showed the presence of grains over the cytoplasm and nuclei of the inoculated cells. In addition, immunofluorescent tests confirmed the presence of viral antigens in the cultures. Antigen was demonstrated as early as six hours post-infection while the control cultures were negative for the length of the experiment. Electron microscopic examination of VSW cells 72 hours post-infection showed the presence of tubular structures, interpreted to be abnormal viral capsids.

The unconfirmed results of Himeno and co-workers (1967) and of McIntosh and Shamy (1975) are the only cases in the literature in which replication of a baculovirus in vertebrate cells has been demonstrated. The majority of such studies conclude that replication does not occur. The fact that virus can be recovered from vertebrate cell cultures four weeks and three cell passages after the infection (McIntosh and Maramorosch 1973) could indicate that low levels of viral replication do occur. This is certainly a question which needs to be answered before these agents are widely dispersed. Though overt symptoms do not appear in animals other than the target insect, infection by a baculovirus may result in the virus entering an "occult" stage. It may be harbored in the host cells for months or years at undetectible levels. The viral genome may be integrated into the host chromosomes, analogous to many tumor-causing viruses. The consequences of such events are impossible to determine. However, if they are shown to occur in mammals, the possible harm far outweighs the benefits which might be derived from the use of the agents. A more in-depth study of baculovirus
replication in vertebrate cells is necessary before these viruses are considered safe.

DNA-DNA Hybridization to Study Baculovirus Replication in Mammalian Cells

A solution to the question of baculovirus replication in vertebrate cells can be obtained using DNA-DNA hybridization techniques. As of this writing, no such experiments have appeared in the literature.

DNA-DNA hybridization techniques are based on the fact that complementary strands of DNA, dissociated by heat, alkali, or denaturing chemicals, will reassociate at a rate dependent upon their concentration (for review see Britten and Kohne 1968). Under standard conditions of sodium and phosphate concentration, temperature, viscosity, and DNA fragment size, the concentration of a particular DNA molecule can be determined relative to that of a standard of "known" solution by comparing their rates of reassociation. With this information and the size of the DNA molecule, the number of "copies" of a particular DNA molecule can be determined.

In the experiments presented here, the fate of Autographa californica nuclear polyhedrosis virus, following infection of the Vero line of African Green Monkey kidney cells, was determined. Using DNA-DNA hybridization, the number of viral genome copies per cell at various times following inoculation were quantitated. The result was an estimation of the replicative properties of A. californica NPV in mammalian cells.
MATERIALS AND METHODS

Cells and Media

The TN-368 cell line, developed from the ovaries of the virgin adult cabbage looper, *Trichoplusia ni* (Hink 1970), was provided by Dr. M. D. Summers (Texas A & M University, College Station) and was used in this study to propagate infectious virus and polyhedra. Cells were maintained in 25 cm² plastic tissue culture flasks (Corning Scientific Products Division, Corning, N.Y.) at 27°C. The cells were subcultured by diluting 0.5 ml of a 48 to 72 hour old culture into 4.5 ml fresh insect cell culture medium. The medium was prepared by combining 500 ml Grace's insect medium, 17 ml lactalbumin hydrolsate medium, and 12 ml 25% fresh yeast extract (all products of Grand Island Biological Co., Grand Island, N.Y.). The medium was supplemented with fetal bovine serum to 10% and a solution of antibiotics yielding 50 I.U. Penicillin/ml, 50 µg Sterptomycin/ml, and 2.5 µg Fungizone/ml (Flow Laboratories, Inglewood, Ca.).

The Vero line of African Green Monkey kidney cells was obtained from Dr. W. Meinke (University of Arizona, Tucson) and was used to test the possibility of insect virus replication in mammalian cells. The cells were maintained on 100 mm plastic petri dishes (Falcon Plastics, Oxnard, Ca.) in Dulbecco's modified Eagle's medium (Flow Laboratories, Inglewood, Ca.) containing 10% fetal bovine serum and the same antibiotics as above. Cells were grown at 37°C in a humidified CO₂ atmosphere.
Virus

The nuclear polyhedrosis virus of the alfalfa looper, *Autographa californica* (Vail et al. 1971) was tested for the ability to replicate in mammalian cells. The original inoculum was provided by Dr. M. D. Summers and was used to infect TN-368 cultures for the production of supernatant containing non-occluded virus. Monolayers of TN-368 cells were infected at multiplicities of 1 to 10 and incubated at 27°C for two to four days. Cell culture fluids were removed and cellular debris and polyhedra were pelleted at 10,000 x g for 20 minutes. The NOV-containing supernatants were pooled, filtered through Millex 0.22 µm disposable filters (Millipore Corp., Bedford, Mass.), and stored at 4°C.

Infectious virus was quantitated by the 50% end point dilution method (TCID$_{50}$) of Reed and Muench (1938) on TN-368 cells in plastic multiwell dishes (Costar, Cambridge, Mass.) as previously described by Vaughn and Stanley (1970).

**Infection of Vero Cells with NPV**

Monolayers of Vero cells were infected 24 hours after they reached confluence. Medium was removed and cultures infected with 0.5 ml of NPV (5 x 10$^6$ pfu/ml) suspended in Dulbecco's modified Eagle's medium. Virus was allowed to absorb for 90 minutes at 33°C. Following infection, monolayers were overlayed with 10 ml of the medium containing 5% fetal bovine serum. Cultures were maintained at 33°C for various time periods prior to extraction of total cellular DNA.
Isolation of Total Cellular DNA

At selected time points after infection, total cellular DNA was extracted from infected Vero cultures by a modification of the procedure of Meinke, Goldstein and Hall (1974). Medium was removed from the infected cultures, monolayers were washed twice with cold phosphate buffered saline, and cells were lysed by the addition of 1 ml of a solution of 8 M urea, 1% SDS, 0.001 M EDTA, 3% isoamyl alcohol, and 0.2 M sodium phosphate buffer (pH 6.8). Viscous lysates were pooled and placed in a Waring blender and homogenized at maximum speed for one minute.

The lysate was applied to a column of hydroxyapatite (4 x 2.5 cm) equilibrated with a solution of 8 M urea, and 0.2 M sodium phosphate buffer (pH 6.8) at room temperature. The column was washed with 150 ml of 8 M urea, 0.2 M sodium phosphate buffer (pH 6.8) at room temperature. The column was then washed with 200 ml of 0.12 M sodium phosphate buffer (pH 6.8) to remove the urea. The temperature of the column was increased to 60°C by a temperature controlled circulating water bath and subsequently washed with 100 ml of 0.14 M sodium phosphate buffer (pH 6.8). DNA was eluted by washing the column with 0.48 M sodium phosphate buffer (pH 6.8).

DNA was phenol extracted once with an equal volume of redistilled phenol. The DNA solution was diluted to 0.12 M sodium phosphate buffer (pH 6.8) with water and sheared to an average piece size of 570 nucleotides (single stranded) in a Ribi cell fractionator at 50,000 pounds per square inch at 4°C. The sheared DNA solution was applied to a second hydroxyapatite column equilibrated with 0.14 M sodium phosphate buffer (pH 6.8).
buffer (pH 6.8) at 60°C. The column was washed with the same buffer until the absorbance of the effluent was less than 0.02 at both 260 and 280 nm. DNA was then eluted with 0.48 M sodium phosphate buffer (pH 6.8). The DNA solution was dialyzed against 0.001 M EDTA (pH 7.0) four times followed by 0.001 M EDTA (pH 7.0) at 4°C. Solutions were quantitated, then lyophilized.

Production and Isolation of Polyhedra

TN-368 cells were infected with non-occluded A. californica NPV by suspending them in a viral suspension at a multiplicity of infection of 1 to 10 for 60 minutes at 27°C. The infected cells were subsequently plated at near confluency and incubated at 27°C for five days. In some cases, tritiated thymidine was added to the cultures at 2 to 4 μCi/ml 24 hours after infection in order that the polyhedra could be followed through the isolation procedure.

Infected cells were lysed by sonication using a Sonifer cell disrupter (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) at a setting of 75 watts for 30 to 45 seconds. Polyhedra and cellular debris were pelleted at 10,000 x g for 30 minutes. Pellets were resuspended in 0.01 M Tris buffer (pH 7.4), 0.01 M EDTA, 1 M NaCl and layered onto 43 to 56% (wt/wt) neutral sucrose gradients prepared in the same buffer. Polyhedra were banded at 20,000 rpm for two hours in the SS-90 vertical rotor and the visible polyhedral band was collected. Polyhedra were washed free of sucrose by pelleting three times through 0.01 M Tris buffer (pH 7.4), 0.01 M EDTA and were stored at 4°C.
Purification of NPV DNA

Viral DNA was purified directly from polyhedra as described by Summers and Anderson (1973). Purified polyhedra were pelleted at 10,000 x g for 20 minutes and resuspended in 0.07 M NaCl, 0.03 M Na₂CO₃, 0.001 M EDTA (pH 10.8). The suspension was warmed to 60°C and allowed to react for 20 minutes. SDS and EDTA were added to 3% (wt/vol) and 0.002 M, respectively. After five minutes the DNA suspension was cooled to room temperature and adjusted to neutral pH. Ethidium bromide was added to 300 μg/ml and the sample was layered onto performed cesium chloride gradients (average density 1.60) prepared in 0.01 M Tris buffer (pH 7.4), 0.01 M EDTA and containing 300 μg/ml ethidium bromide. The DNA was centrifuged in the SW 50.1 rotor for 18 to 24 hours at 33,000 rpm and 25°C. Supercoiled viral DNA was collected from the gradients and ethidium bromide removed by treatment with an equal volume of cesium chloride (o = 1.50) saturated isopropanol. DNA solutions were dialyzed extensively prior to use.

In Vitro Labeling of NPV DNA

The methods of labeling DNA in vitro were similar to those described by Lancaster and Meinke (1975). Supercoiled viral DNA was treated with pancreatic DNAse I in 0.05 M Tris buffer (pH 7.4), 0.005 M KCl, 0.005 M MgCl₂ at a ratio of 100:1 (wt/wt) at 37°C for 65 minutes. DNAse was inactivated by heating to 60°C for 15 minutes.

DNAse treated DNA was then labeled in vitro with Escherichia coli DNA polymerase I under conditions of repair synthesis (Kelly et al. 1970). Viral DNA (0.6 μg) was suspended in a volume of 1 ml containing
0.07 M potassium phosphate buffer (pH 7.4), 0.007 M MgCl$_2$, 0.001 M 2-mercaptoethanol, and 0.002 M of each of the necessary unlabeled deoxy-nucleotide triphosphates (dNTP). The DNA was labeled with $^3$H TTP (53.1 Ci/mmol) at 1300 pCi/µg DNA. Synthesis was initiated by addition of 20 units of *E. coli* DNA polymerase I per microgram of DNA. The reaction mixture was incubated at 15°C for 60 minutes. Reactions were terminated by addition of sarkosyl to 1%. Labeled DNA was passed through a Sephadex G-50 column (0.7 x 20 cm) equilibrated with 0.02 M Tris buffer (pH 8.0), 0.1% sarkosyl, 0.001 M EDTA. Fractions containing radiolabeled DNA were pooled and stored over chloroform at 4°C. Incorporation of radioactivity into DNA was determined by trichloroacetic acid precipitation of 2 µl DNA samples with 100 µg of tRNA as carrier. The DNA was labeled to a specific activity of approximately $2 \times 10^6$ counts/minute per microgram DNA.

The size of the $^3$H-labeled NPV DNA was determined by sedimentation velocity relative to a standard size DNA. The standard (provided by Dr. W. Meinke, University of Arizona, Tucson) was DNA from a human lymphocyte cell line (Wil 2) which had been labeled with $^{14}$C to a specific activity of 2600 cpm/ng DNA and sheared to a fragment size of 8S. Both the $^3$H-NPV DNA and the $^{14}$C-Wil$_2$ DNA were layered onto 5-20% (wt/wt) alkaline sucrose gradients (pH 13.0) and centrifuged in the SW 50.1 rotor for 10 hours at 35,000 rpm and 15°C. The gradients were fractionated and the size of the $^3$H-NPV DNA was calculated by the distance of peak migration through the gradient, relative to that of the 8S standard.
**DNA-DNA Hybridization**

DNA solutions were denatured in 0.48 M sodium phosphate buffer (pH 6.8), 0.05% SDS, 0.001 M EDTA by heating to 105°C for five minutes in sealed 50 μl micropipettes. Denatured DNA was allowed to reassociate at 65°C. At various times samples were removed and diluted in 2.0 ml of 0.14 M sodium phosphate buffer (pH 6.8), 0.05% SDS. Single-stranded DNA was separated from reassociated duplex DNA by hydroxyapatite chromatography using a 10 x 15 mm column of hydroxyapatite maintained at 60°C (Britten and Kohne 1968). Single-stranded DNA was eluted from the column with five 2.0 ml washes of 0.14 M sodium phosphate buffer (pH 6.8), 0.05% SDS, and reassociated DNA was subsequently eluted with three 2.0 ml washes of 0.48 M sodium phosphate buffer (pH 6.8), 0.1% SDS. Radioactivity was determined for each wash by addition of 15 ml of a toluene-Triton x-100 (2:1 vol/vol) scintillation fluid. Immediately after denaturation, less than 10% of in vitro labeled DNA was eluted as double-stranded DNA on hydroxyapatite columns.

**Chemicals**

Tritiated thymidine (specific activity 50 Ci/mmol) and [3H] TTP (specific activity 53.1 Ci/mmol) were purchased from New England Nuclear Corp. dATP, dGTP, and dCTP were purchased from Biogenics Research Corp. Pancreatic DNAse I was purchased from Sigma Chemical Corp. *Escherichia coli* DNA Polymerase I was purchased from Boehringer Mannheim Corp.
RESULTS

Isolation of Viral Inclusion Bodies

The nature of this study dictated that viral DNA be isolated from virus which had been packaged in viral inclusion bodies (polyhedra). Polyhedra were released from infected cells by sonication and separated from non-occluded virus and cell debris by sucrose gradient velocity sedimentation (Figure 4). The large peak seen in fractions 10 through 15 corresponded to a clearly visible white band in the gradients. The band was confirmed to contain polyhedra, in the absence of appreciable quantities of cells or debris, microscopically.

Isolation of Viral DNA

Viral DNA was released directly from occluded virus by combining alkaline solubilization of polyhedra with SDS denaturation of viral proteins (see Materials and Methods). DNA thus released was banded by density in cesium chloride-ethidium bromide gradients (Figure 5). The supercoiled viral DNA, contained in fractions 8 through 11, was collected and treated as described in Materials and Methods to remove intercalated ethidium bromide prior to use.

In Vitro Labeling of NPV DNA

Supercoiled NPV DNA molecules were labeled with $^3$H-TTP as described in Materials and Methods. The course of the labeling reaction was followed by measuring the TCA-insoluble radioactivity of small
Figure 4. Isolation of polyhedra from A. californica NPV-infected TN-368 cells by sucrose gradient sedimentation—sedimentation was from right to left.
Figure 5. Isolation of supercoiled *A. californica* NPV DNA by CsCl/EtBr density sedimentation
samples taken at 15 minute intervals. The data presented in Figure 6 show the rate of incorporation of ^3H-TTP into NPV DNA. After 60 minutes ^3H-NPV DNA had reached a specific activity of 2 x 10^6 counts per minute per microgram of DNA.

After terminating the reaction, ^3H-NPV DNA was separated from free nucleotides by passing it through a Sephadex G-50 column (Figure 7). The first radioactive material eluted from the column (fractions 5-10) represents ^3H-NPV DNA while the second, larger peak represents unincorporated nucleotides.

The average piece size of the ^3H-NPV DNA molecules was determined by their rate of migration through an alkaline sucrose gradient relative to an internal 8S, ^14C-labeled DNA standard (Figure 8). The ratio between the sizes of the two molecules is directly proportional to the ratio between the distances each molecule migrated through the gradient. Thus, ^3H-NPV DNA was determined to have a sedimentation coefficient of 7.4S relative to the 8S standard. This corresponds to an average single stranded length of about 550 nucleotides.

**Fate of NPV DNA in Vero Cells**

DNA-DNA hybridization techniques were used to determine the number of NPV genome copies present in Vero cells at various times following infection with the virus (see Materials and Methods). The rates of reassociation of a ^3H-labeled NPV DNA "probe" in the presence of NPV-infected Vero DNA are shown in Figure 9. Reassociation of the ^3H-NPV probe in the presence of uninfected Vero DNA established the baseline reassociation rate. The higher slopes of the other curves in Figure 9
Figure 6. Rate of $^3$H-TTP incorporation into A. californica NPV DNA
Figure 7. Sephadex G-50 isolation of $^3$H-labeled *A. californica* NPV DNA—elution was from left to right.
Figure 8. Alkaline sucrose gradient sedimentation of $^3$H-labeled *A. californica* NPV DNA relative to an $8S$ $^{14}$C-labeled DNA standard—sedimentation was from right to left.
Figure 9. Reassociation of $^3$H-labeled *A. californica* NPV-infected Vero DNA—(-O-) uninfected Vero control, (- -) Vero DNA extracted at 0 hours post-infection, (- -) 4 hours post-infection, (-O-) 24 hours post-infection, (- -) 48 hours post-infection
reflect increased rates of reassociation of the $^3$H-NPV probe due to homologous NPV DNA sequences present in Vero DNA at the post-infection time points indicated. Factors of increased reassociation rate were determined from these curves for each of the time points and are listed in Table 1.

To determine the number of NPV genome equivalents per diploid cell at each of the time points, the equation of Gelb, Kohne and Martin (1971) was used:

$$\text{NPV gen. eq./Vero cell} = \left( \frac{3\text{H-NPV gen. eq./cell} \times \text{factor of increased rate}}{\text{NPV gen. eq./cell}} \right) - 3\text{H NPV gen. eq./cell}$$

The factor of increased reassociation rate was determined from the data shown in Figure 9. The number of $^3$H-NPV genome equivalents per Vero cell in each reassociation reaction was determined as follows:

$$3\text{H-NPV gen. eq.} = \frac{\text{mol. wt. Vero DNA}}{\text{mol. wt. NPV DNA}} \times \frac{\text{conc. (ug/ml) NPV DNA in rxtn}}{\text{conc. (ug/ml) Vero DNA in rxtn}}$$

The estimated molecular weights of Vero and NPV DNAs are $3.92 \times 10^{12}$ daltons and $9 \times 10^7$ daltons, respectively (Gelb et al. 1971, Summers 1975). The concentrations of NPV-infected Vero DNA and $^3$H-NPV probe used in each experiment are listed in Table 1.

As the data in Figure 9 and Table 1 show, the rate of reassociation, and therefore the number of NPV genome equivalents per Vero cell, decreased with time post-infection. The data shows that an average of 2.26 NPV genomes were present in each cell immediately following infection. In four hours this number was reduced almost in half to 1.33.
<table>
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<th>Hours Post-Infection</th>
<th>Conc. (μg/ml)</th>
<th>Factor of Increased Rate&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NPV Genome Equivalents per Diploid Cell&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% Viral DNA Remaining</th>
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<td>^3H-labeled NPV DNA</td>
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<sup>a</sup>Factor of increased rate of reassociation over that of the control

<sup>b</sup>Calculated by method of Gelb et al. (1971) with molecular weight of NPV DNA at 90 x 10<sup>6</sup> (Summers 1975)
By 24 hours only 0.31 genome equivalents per cell remained. At 48 hours post-infection the number was reduced to 0.10 NPV genome equivalents per cell. In terms of percentage, the amount of viral DNA in the cells had been reduced to 4.4% of the original amount in 48 hours. In Figure 10 the decline in NPV genome equivalents per cell over time is presented.
Figure 10. Rate of *A. californica* NPV DNA loss from infected Vero cells
DISCUSSION

The results of this study demonstrate two things; first, that the majority of NPV genetic material was rapidly eliminated from Vero cells shortly after entry. Second, that this elimination was either inefficient or the virus countered the elimination process through replication or by the integration of its DNA into host cell chromosomes.

The fact that the viral DNA was lost from the cells is apparent in the data presented in Table 1 and Figure 10. During the first four hours following infection, over 40% of the original viral DNA disappeared from the cells. After 24 hours, more than 85% had disappeared.

From 24 to 48 hours post-infection the amount of viral DNA in the cells continued to decline, although at a much slower rate. In the first 24 hours it was reduced to one-eighth the original input, while in the second 24 hours it was only reduced by an additional one-third. If the viral DNA was being degraded by the cell, the rate of decline of the viral genome equivalents per cell would have demonstrated second order kinetics. If this had been the case, the semi-log plot in Figure 10 would have been a straight line. However, the data yielded a curved line, indicating a degree of stabilization in the amount of viral DNA remaining in the cells after 48 hours. How the DNA was stabilized is subject to interpretation.

Himeno et al. (1967) have shown that polyhedra, containing viable NPV, were produced in human amnion cells which were infected
with naked viral DNA. These findings indicated that the viral DNA was not only harbored in these cells, but was also replicated and transcribed into message. In another study, McIntosh and Shamy (1975) used fluorescent antibody to show that NPV-coded proteins were produced in NPV-infected viper cells as early as six hours post-infection.

It is obvious from the data presented here that NPV does not replicate to any great extent in Vero cells. However, the lack of second order kinetics in the rate of decline of viral DNA implies that a low level of viral DNA replication or chromosomal integration may have occurred which could only be detected when the viral DNA reached a low enough concentration in the cells. Further studies are required to determine whether the viral DNA levels at later time points remain constant, indicating a persistent or integrated state, or if they increase, indicating continued viral DNA replication.

The data presented here could be interpreted to mean that once in the cells, some of the virus was simply not uncoated. This would result in the same non-linear curve as seen in Figure 10. Even if this were the case, the results would indicate an unusual relationship between an insect virus and a mammalian host.

McIntosh and Maramorosch (1973) have demonstrated that NPV could be recovered from inoculated cultures of several types of vertebrate cells for up to four weeks following infection. However, they concluded that viral penetration occurred at very low frequency and that viral replication did not occur in these cells. The assumption was that most of the virus merely adhered to the cell's surfaces.
throughout the experiment. In this study, cells were treated to remove any membrane adhering virus. The NPV-infected cells were trypsinized and washed several times with phosphate buffered saline prior to the extraction of total cellular DNA.

In these experiments, the Vero cells were confluent when infected. They should have been inhibited from further cell division. However, it is quite common for cells to undergo further divisions when old medium is replaced with fresh, as was done in these infections. Cell counts were not maintained during the experiment, so it is possible that the cells did undergo further divisions after the infection. If this did occur, the data presented here would be misleading in that the concentration of cellular DNA would have increased relative to the concentration of viral DNA added to the culture. The result would be an artificial dilution of the number of viral genome equivalents per cell. The percent of viral DNA remaining in the cells at the later time points would be much higher than indicated. Thus, the deviation from second order kinetics would be even more exaggerated than the data indicates. The bearing this would have on the interpretation of these data would be to further confirm the belief that viral DNA replication, integration, or persistence occurred in these cells.
REFERENCES


