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DEVELOPMENT AND STANDARDIZATION OF IDENTIFICATION AND MONITORING TECHNIQUES FOR BACULOVIRUS PESTICIDES

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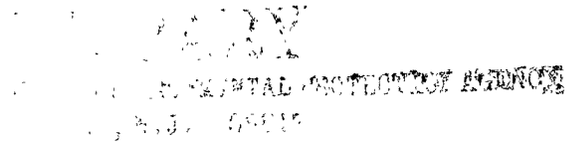

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FOREWARD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis, and the toxicology of pesticides as well as other chemical pollutants. The Laboratory develops and revises air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is preparing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The majority of the registered pesticides are chemical agents. A few, however, are biological in nature because the active ingredients are microbial. Of these microorganisms, viruses are perhaps the most unique in structure, biology, and the intimacy of their parasitic relationship with their hosts. These proceedings consider whether potential biohazards to human health and other biological components of the environment exist when insect viruses are used as pesticides and whether such potentials have been adequately assessed in view of our current knowledge of these agents.

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Abstract

Biological pesticides, in particular the microbial formulations, are fundamentally different from chemical pesticides in the nature and mode of action of the active agent. The pesticidal action is dependent on the activities of living organisms. Identification, detection and monitoring methods for biological pesticides, because of their nature and characteristics, are divergent from those classically associated with chemical toxicants. Therefore, a new class of standardized, specific and sensitive methods must be developed. Fortunately, much of the sophisticated technology exists in the area of medical microbiology and can be immediately adapted and applied to microbial pesticides. Any difficulties encountered in adapting this technology will be the result of our lack of knowledge about the biology of the organisms.

One objective of this grant was the development, adaptation and application of specific sensitive diagnostic and clinical techniques for identification, detection and monitoring of viral pesticides. A portion of this research was also commited to study some of the basic biology and characteristics of baculoviruses so that a more thorough understanding of the limitations of the developed monitoring technology would be better understood. This technology can now be applied for the assessment of health and ecological effects, as well as for regulatory concerns.

A. Baculovirus identification:

1. By analysis of the structural polypeptides of enveloped nucleocapsids by analytical SDS-polyacrylamide gel electrophorens (SDS-PAGE) baculovirus species can be differentiated. This is one tool for identification which can be used with reliability and routinely, since different species of baculoviruses contain several different structural polypeptides. However, MNPVs,

i.e., those baculoviruses with more than one nucleocapsid per envelope, reveal qualitative and quantitative differences when the singly enveloped nucleocapsids are purified from the multiples and each are analyzed by SDS-PAGE. Such variation might be misinterpreted as strain differences if it was not known which biological form of the virus was being analyzed.

Furthermore, our biological studies have shown that there are two infectious forms of a given species of baculovirus which occur during the infection cycle. Most studies are conducted on the alkali-liberated from because it is easily obtained in sufficient quantities for analysis. The non-occluded or extracellular virus is not occluded during the infection cycle, is 200 fold more infectious than the alkali released form and has different neutralizing antigens. Although this form of virus can be easily identified by SDS-polyacrylamide gel electrophorens, care has to be taken when using the appropriate serological identification technique or it will not be detected.

Polyhedrins and granulins have been purified and identified by two-dimensional peptide mapping. Although individual baculoviruses can be identified by this technique the nature of the experimental procedure is too complex to allow routine application for identification.

2. Serological Assays for identification and detection.

Radioimmuneassay (RIA) has been developed for the quantitative detection of the major structural protein of baculoviruses, polyhedrin or granulin. By polypeptide mapping (above) and serological comparisons, it is now known that polyhedrins and granulins are related and, therefore, group antigens. Standardized

RIA as developed is able to detect 70-80 picograms of polyhedrin protein which is equivalent to approximately 40-50 polyhedra. This assay can significantly detect polyhedrin in as few as 100 infected cells as early as 12 hours psot-infection in susceptible cells.

3. RIA for polyhedrin can be useful for monitoring the levels of polyhedra applied in environmental samples. The limitations for the use of this assay are:

    ]) polyhedrins are group antigens and not virus specific as analyzed by RIA;

    2) although the RIA is good for a known or standard system where polyhedra are routinely produced, it is now known that polyhedrin is not required for infection nor need be synthesized in cells with replicating virus.

Also, the synthesis of polyhedrin is dependent upon the cell, tissue or culture conditions of the assay. Therefore, the assay cannot be reliably used to detect replicating virus, especially in non-target hosts.

Although RIA has been developed and standardized for polyhedrin, there are limitations for the assay which limits its use for identification and detection. We only discovered those limitations by these studies. Therefore, emphasis is now being given to RIA analysis of virus structural proteins to identify more reliable virus specific proteins for serological detection.

Immunoperoxidase has been developed and standardized as an alternative method for detecting the presence of virus antigens

in cell and tissues. The assay is very sensitive to being able to detect polyhedrin antigens 2-5 hours prior to visualization with the phase microscope. Antisera against replicating virus detected those antigens in cells 2-4 hours before infectious viruses could be assayed. The technique is also sensitive to the level of positive detection of 1 in 100 infected cells and can detect replicating virus in the absence of polyhedrin synthesis. This technique can be easily adopted to screen a variety of tissues and cell types in routine monitoring procedures.

4. Restriction endonuclease enzymes are an additional and powerful tool for identification and for genetics studies. This technology has been standardized for baculoviruses and every baculovirus isolate studied to date shows specific genetic markers for identification. Although serology is useful for clinical applications, standard monitoring by serological means will not given an accurate picture of genetic stability or change.

By cloning we have shown that the wild isolates of baculovirus of Autographa californica is a mixture of genetic variants. We do not know what this means concerning mutation or recombination or what biological significance can be attributed to host specificity. The genetic stability of these viruses must be understood and such is essential if a realistic assessment is to be made for potential ability to infect non-target organisms and for safe use.

B. Detection and monitoring

The structural proteins of baculoviruses are being identified in order to develop and apply highly specific and sensitive seriological detection methodology. RIA and immunoperoxidase assays are available for polyhedrins and granulins. The conditions for extending these assays to other baculoviruses have been studied so that development of additional assays could be easily accomplished at the replicating virus level. However, such assays have limitations due to sensitivity and should be used along with assays specific for the genetic material of the virus.

The use of restriction endonuclease enzymes for the specific identification of the structure of baculovirus genomes has been applied in these studies with good success. This procedure can detect genetic changes in the virus after exposure to target and nontarget systems. Furthermore, the methodology as developed with this project is the first step for the development of molecular hybridization technology. Molecular hybridization with nucleic acid probes can be used for detection of less than 0.2 genomes per cellular DNA equivalent in non-target organisms. Therefore, this can be used for monitoring for persistence and integration in non-target organisms, both activities of which are essential to evaluate one potential role of baculovirus genomes to act as carcinogens.

C. Baculovirus Biology

The biological activity of baculoviruses has been shown to exhibit complex behavior in the susceptible host and in tissue culture. There are two infectious forms which have different roles and fates during invasion and infection: the polyhedral form is responsible for persistence and transmission in the environment, and the extracellular virus which is not included in the polyhedra and is responsible for secondary infection of

host tissues. Extracellular virus is 2.2 to 2.4 x 10^3 more infectious than the alkali released form (from polyhedra), has a different structural protein composition and different neutralization antigens. These significant biological and structural differences reveal why it is necessary for detection and monitoring that the appropriate diagnostic technique is used. This reveals the importance of understanding the biology of the virus and, by knowing this, how to evaluate and assess biological impact. For example, when evaluating health assessments the results of these studies suggest that it would be most appropriate to use the most infectious form of the virus.

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SECTION I

INTRODUCTION

The use of insect pathogens, in particular viruses, as a viable alternative or aid in pest control programs has been comprehensively documented in the published proceedings of several conferences since 1973 (FAO Report, 1977; WHO Technical Report Series 531, 1973; Summers et al., 1975; Summers and Kawanishi, 1978). Baculoviruses have been given favorable attention and consideration for development once a comprehensive evaluation of safety is documented and reconfirmed (NAS Report, 1975: pp. 19-20). Chemicals are the major defense for the suppression and control of insect pests. However, recent problems associated with undesirable side effects are now pointing to the urgency for the development of new or modified methods for arthropod control; especially highly specific methods for economically important and dangerous species (FAO Report, 1977; WHO Technical Report Series 585, 1976). It is important that alternative methods do not cause persistent or cumulative toxic contamination of the environment and, perhaps most importantly, methods must be utilized against which resistance (biochemical and/or behavioral) is not readily developed. As long as insecticides remain the principle tool for control of agriculturally important insect pests and vectors of human disease, it is likely that resistance will continue to be the major threat and perhaps final difficulty (FAO Report, 1977; WHO Technical Report Series 585).

The number of really useful economic insecticides is limited and new compounds or compounds with novel modes of action do not appear to be forthcoming in quantity. The appearance of increasing resistance to new alternative chemicals makes the situation apparently worse. The potential advantages of viral pesticides over chemicals are: natural occurence in the environment, specificity, low toxicity, little or no residue, perhaps the least likelihood of inducing resistance (this has not been properly tested) and, in some cases, the ability to establish and maintain persistent infectious forms in the environment.

With the projected problems associated with world food production and public health the use of microbials for pest control with specific and novel modes of action may be a new and important approach (Arrata, 1977). EPA has registered two viral pesticides: a formulation of the nuclear polyhedrosis virus (NPV) of Heliothis zea and NPV of the Douglas Fur Tussock Moth (Hemerocapsa pseudosugata). The Gypsy Moth NPV (Porthetria dispar) is expected to be registered shortly. Recent reports reveal that with these registrations the use of microbials on a worldwide basis is expanding rapidly from an experimental situation to more routine uses for applied problems (Harrap, 1977, Falcon, 1977).

It seems logical, however, to document the potential problems as well as benefits for the use of baculoviruses in order to provide the appropriate forum and background for evaluating possible undesirable consequences or appropriate risks (Tinsley and Melnick, 1974; Summers et al., 1975; Summers and Kawanishi, 1978). The testing procedures and criteria presently used for registration of baculovirus formulation

have followed those tests utilized for the registration of chemical pesticides. It has been clearly identified in the above documents that those tests need to be modified for invasive and/or replicating entities such as viruses. Tests more appropriate for the detection of replication or persistence in physical and biological systems are badly needed. For this specific and sensitive detection and monitoring technology needs to be available.

However, basic to this are other problems which have been fully addressed (see proceedings of conferences cited above) but not implemented. Basic to the appropriate detection technology is the use of reliable identification technology. In fact, identification has been based primarily upon susceptibility and host range for a given baculovirus. Identification and cataloguing of baculoviruses by genetic or serological means has not been established and used. As a result events such as mutation or recombination cannot be evaluated because of the lack of appropriate markers. Further, little if anything is known about the genetics of baculoviruses (Summers and Kawanishi, 1978). It is shown that baculovirus genomes are large molecules of approximately 10^8 suggesting that they have a genetic capability comparible for example to the T-even bacteriophages, Herpes and cytomegaloviruses.

Basically, if baculoviruses are to be evaluated in terms of safe use, degree of risk by use, or the possibility of there being a risk beyond of which we are aware, it is necessary to study the structure and stability of baculoviruses with the appropriate techniques to see if they do change as they evolve subject to environmental pressures, If they do change, what would be the nature and extent of that change?

The ability of a virus to change usually involves mutation or genetic recombination for each of which there may be several potential mechanisms. It is quite clear by various mechanisms that vertebrate viruses with large genomes, such as herpes, adeno, and cytomegaloviruses (CMV) undergo change by recombination. With baculoviruses at present it would be difficult to document or establish the difference between recombination and mutation without being able to identify whether a new hybrid virus had either a rearranged or new set of genes. Also, certain baculoviruses contain many nucleocapsids common to a viral envelope. Little is known about whether or not these represent multiple genomes within a single bundle of particles or whether this is in fact numerous copies of the same genome. Such combination(s) make it difficult to predict possible relationships involving defective particles, recombination, and etc. This may be quite important since it is fairly well documented that mixed viral infections can occur in nature with insect viruses. Perhaps of potentially greater importance is possible recombination between a baculovirus and host or unrelated viral DNA. Furthermore, we know nothing about the frequency by which such a genetic change may occur, since the genomes of our baculoviruses have not been properly mapped using the appropriate techniques. Once again these kinds of studies cannot be conducted until the appropriate standards and markers have been utilized for identification and comparison.

Direct studies identifying the structural properties and anatomy

of baculovirus DNAs are important. However, it must be recognized that it is difficult to directly relate biological function to genetic maps. Therefore, studies on virus structural proteins will also be important in order to make comparisons between proteins which are specific or contain group associated antigenic properties. This is important to develop the basis for routine serological identification and detection.

Basic to all of this is another rather concrete realization. Baculoviruses will likely not be used as individual control agents in pest management applications. The most effective application for insect viruses is in integrated pest management schemes. This means that baculoviruses will be applied along, or in conjunction, with reduced concentration of chemical pesticides, other pathogens such as bacteria or fungi, or in combination with other organisms such as parasites and predators. In particular, little is known about the combined activities of chemical pesticides or related agents as combinated with viral replication: little is known about mutagenic potential of chemical pesticidal agents used in combination with baculovirus.

SECTION 2

CONCLUSIONS

Because of the structural complexity and biological differences between extracellular and occluded virions, it is important that identification and monitoring technology be developed for both forms until the reasons for these biological differences are better characterized. It is important to understand better which form is potentially the most infectious to non-target organisms. The development of serological identification and monitoring technology for baculovirus granulins and polyhedrins has been accomplished and applied. Although the technology is extremely sensitive, it is presently believed with new data at hand that RIA for granulins and polyhedrins will not be all that useful from the standpoint of reliable monitoring and identification. Development of RIA for virus specific proteins which can directly monitor for virus specific antigens and, therefore, are more direct indicators of viral infection should be developed. In addition to radioimmunoassay, immunoperoxidase and neutralization techniques have been applied to the investigation and detection of baculovirus antigens in infected cells. Preliminary studies have revealed while isolates of baculovirus are mixtures of genotypic and phenotypic variants the significance and/or stability of this variation as it exists in nature is not well understood relative to the genetic stability of baculoviruses in terms of mutation and/or recombination.

SECTION 3

RECOMMENDATIONS

It is recommended that for future studies on baculoviruses that plaque-purified and genetically identified and defined isolates be utilized as the basis for evaluating genetic and genomic stability relative to mutation, recombination, and/or change with time by passage through host and alternate host systems. It is necessary to develop identification and detection technology and both the genomic and phenotypic levels in order to provide screening technology for evaluating safe and effective use of baculoviruses in the biological and physical environment. For serological studies it is important that viral specific structural polypeptides be identified for the development and application of radioimmunoassay technology. It is suggested that individual structural polypeptides be isolated and evaluated in terms of the specificity of their antigenic reactions and that this should be done for both the extracellular virus and nonoccluded virion forms.

SECTION IV

METHODS AND MATERIALS

Viruses. Baculoviruses used in this study were: Autographa californica (AcMNPV), Rachiplusia ou (RoMNPV), Anticarsa gemmatalis (AgMNPV), Heliothis armigera (HaMNPV), Trichoplusia ni (TnSNPV), and Heliothis zea (HzSNPV), nuclear polyhedrosis viruses; Trichoplusia ni (TnGV) and Spodoptera frugiperda (SfGV), granulosis viruses. AcMNPV, RoMNPV, TnSNPV, and TnGV were produced by infecting T. ni larvae with the appropriate polyhedra. SfGV was produced in Spodoptera frugiperda larvae. HaMNPV and HzSNPV and the original inoculum of RoMNPV were kindly provided by C. Y. Kawanishi (EPA, Research Triangle Park, N. C.) and J. Hamm (USDA-ARS, Tifton, Ga.), and AgMNPV, by G. Allen (University of Florida, Gainesville, Fla.).

Isolation of polyhedra. Polyhedra were isolated essentially as reported (Summers and Smith, 1976), with certain modifications: Polyhedra purified by banding on sucrose gradients were suspended in 0.01 M Tris (pH 7.8), 0.01 M EDTA, 1.0 M NaCl and were stirred for 30 min at room temperature. The polyhedra suspension was layered on 40 to 63% w/w continuous sucrose gradients in 0.01 M Tris buffer and was centrifuged at 100,000 g for 30 min. The polyhedra band was removed and washed free of residual sucrose by differential centrifugation and then was resuspended in 0.01 M Tris (pH 7.8) 0.01 M EDTA.

Virus purification. Virus particles were removed from purified polyhedra by incubating the polyhedra for 60 min at 0-4 in 0.1 M Na_2CO_3, 0.01 M EDTA, 0.17 M NaCl, pH 10.9 (Summers and Smith, 1975). The preparations were layered on sucrose gradients buffered in 0.1 M Tris (pH 7.8), 0.01 M EDTA (ranging in density from 1.17 to 1.25 g/ml) and were centrifuged for 1 hr at 24,000 rpm (SW-27) at 4°. The bands of virus were removed from the gradients and the virus particles were washed free from sucrose by differential centrifugation and were resuspended in water and stored at -70°. With AcMNPV, AgMNPV, RoMNPV, and HaMNPV which have one or multiple nucleocapsids per envelope (M = more than one nucleocapsid per envelope; S = enveloped single nucleocapsid per envelope) the top band containing single-enveloped nucleocapsids was easily separated from the other bands (referred to as multiples) which contained more than one microcapsid per envelope particles (Summers and Volkman, 1976). Comparisons of polypeptide composition were made between singles and multiples.

Purification of capsids. Each of the purified virus perparations was adjusted to a final concentration of 500 µg of protein/ml in 0.01 M Tris (pH 7.8), 0.01 M EDTA, 2% NP-40 (v/v) and incubated for 18 hr at 37°. Each preparation was layered on a cesium chloride gradient with a density ranging from 1.18 to 1.55 g/ml and was centrifuged for 2 hr at 20° at 35,000 rpm (SW-41 rotor). The visible bands were removed, pelleted by differential centrifugation, and later utilized for polyacrylamide gel electrophoresis and observation by microscopy. For density determinations, each gradient was fractionated with an ISCO density gradient fractionator and gradient scanning profiles were recorded on an ISCO UA-2 monitor. Densities of the individual samples were determined using an Abbe refractometer.

Purification of polyhedrin. Polyhedrin and granulins were purified using a modification of the procedure reported by Summers and Smith (1976). Highly purified polyhedra were suspended in 0.01 M Tris (pH 7.8), 0.01 M EDTA (a final concentration of 5.0 mg of protein/ml) and incubated for 2 hr at 70° to inactivate the associated protease. Protease-inactivated polyhedra were washed free of buffer by differential centrifugation. To insure further the inactivation of the protease, the polyhedra were resuspended in 0.01 M Tris (pH 7.8), 0.01 M $HgCl_2$ (final concentration of 5 mg of protein/mg) and allowed to equilibrate at room temperature overnight. The polyhedra were removed from the buffer and enzyme inhibitor by pelleting, using repeated differential centrifugation and extensive washing of the pellets with water. Solubilization of the polyhedra was achieved by adding 0.1 M Na_2CO_3, 0.17 M NaCl, pH 10.8, for 19 min at a concentration of 5 mg of protein ml at 4°. The solution was centrifuged at 24,000 rpm (SW 27 rotor) for 30 min at 5° to remove any insoluble material. The solubilized polyhedrins were then utilized for polyacrylamide gel electrophoresis described previously. Granulins and polyhedrins were stored at -70° and were shown to be stable relative to enzyme degradation by polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis and densitometry. Electrophoresis in an 11.0% polyacrylamide vertical gel slab in the presence of 0.1% SDS was conducted as described by Laemmli (1970). The samples were immediately subjected to electrophoresis for 3 hr at a constant power of 7.5 W/gel slab (ISCO Model 493 Constant Power Supply). The gel slab was 14 cm wide, 12 cm high, and 1.5 mm thick. The slabs were fixed in 25% isopropanol, 10% acetic acid, stained with 0.04% Coomassie brilliant blue R-250 for 4 hr, and destained in 10% acetic acid. Stained gels were then photographed and the transparencies were scanned at 600 nm using a Beckman DU spectrophotometer equipped with a Gilford 2400 linear transport accessory.

Molecular weight and protein determination. The method described by Weber and Osborn (1969) was used to determine the apparent molecular weights of the viral proteins as compared to the molecular weights of standard proteins by gel electrophoresis.

The protein determination method of Lowry et al. (1951) was used to estimate the amount of protein relative to bovine serum albumin as the standard.

Cell culture. The continuous cell line of Trichoplusia ni, TN-368, was obtained in the 57th passage from W. F. Hink of Ohio State University in June 1974. Since then the cells have been passed routinely three times per week in disposable plastic tissue culture flasks, with the initial concentration being 1×10^5 to 2×10^5 cells per ml. Daughter cell lines were developed from isolated single cells of TN-368 (Volkman and Summers, 1976) and these cloned lines were handled in the same manner as TN-368. The cultures were grown at 28°C, and the medium used was TNM-FH (Hink, 1970). Antibiotics were not used for routine maintenance of the cells.

Extracellular virus. Initially, AcMNPV was supplied by P. V. Vail and W. F. Hink in the form of infectious tissue culture medium. TN-368

cells infected with this medium yielded polyhedra that were in turn used to infect T. ni larvae for the production of infectious hemolymph. RoMNPV was obtained in the occluded form from C. Y. Kawanishi. T. ni larvae infected with the polyhedra yielded infectious hemolymph, which was used subsequently to infect TN-368 cells for the production of NOV.

Hemolymph used in the neutralization experiments was collected from infected T. ni larvae by removing one or two prolegs and catching the resultant drops of infectious fluid in a test tube containing TNM-FH plus antibiotics (200 µg of penicillin and streptomycin per ml and 5 µg of amphotericin B {Fungizone} per ml).

Preparations of LOVAL used as antigens for generating antisera as well as in neutralization experiments were made in the following way. The virions were liberated from gradient-purified polyhedra by incubating the polyhedra for 10 min at 28°C in 0.1 M Na_2CO_3 + 0.05 M NaCl, pH 10.9 (Summers and Smith, 1975). The preparations were then immediately layered on sucrose gradients made in 0.01 M Tris buffer (pH 7.8)-0.0. M EDTA (ranging in density from 1.18 to 1.25 g/ml) and centrifuged for 2 hr at 24,000 rpm (SW27 rotor). The distinct bands of one through many nucleocapsids per envelope were collected and pooled (Summers and Volkman, 1976). For some experiments, the band containing one nucleocapsid per envelope was kept separate from the rest.

Virus from the fat body of AcMNPV-infected larvae was collected in the following way. The infected fat bodies were removed from five larvae and homogenized in TNM-FH plus antibiotics with a glass homogenizer. The mixture was centrifuged at 2,100 x g for 15 min, and the supernatant fluid was decanted and filtered through a 0.18- to 14-µm microfiberglass filter. The resultant infectious preparation was used in neutralization studies.

Nonoccluded intracellular virus from TN-368-13 was obtained simply by homogenizing a suspension of AcMNPV-infected cells, which had previously been pelleted and rinsed twice, in TNM-FH. The disrupted cell suspension was cleared of heavy and large debris by centrifugation at 4,000 x g for 15 min. The supernatant fluid was used in the neutralization experiment.

Extracellular virus or PMB-NOV was obtained from the culture medium of synchronously infected TN-368-13 (or S. frugiperda) cells in advance of cytolysis 36 to 48 hr after infection (EV will refer to TN-368-13 derived virus unless specifically referred to as otherwise). Cells were pelleted by centrifugation at 300 x g for 15 min (to minimize cell disruption), and the supernatant fluid was further clarified by centrifugation at 4,000 x g for 15 min. This 4,000 x g supernatant fluid constituted the crude virus preparation, which was used in the neutralization experiments. For some studies (e.g., quantification of physical particles and the generation of antisera), the preparation was further purified as follows. The 4,000 x g supernatant fluid was centrifuged at 12,000 x g for 45 min to pellet the virus. The pellets were resuspended in 0.01 M Tris (pH 7.8)-0.01 M EDTA and layered on sucrose gradients in that same buffer, ranging in density from 1.1 to 1.3 g/ml. The gradients were centrifuged at 358,000 x g for 1 hr at 4°C. The

1.17- to 1.18-g/ml region of the gradients containing the virus was collected and held for experimentation (Summers and Volkman, 1976).

Plaque assay of infectious virus. For the plaque assay (Hink and Vail, 1973), (Volkman and Summers, 1975), log-phase TN-368 cells were seeded into 30-mm Corning plates at 3.5 x 10^5 cells per plate in 2 ml of medium. Cells were allowed to attach for 1 to 2 hr. The medium was carefully removed, and the cells innoculated with a 0.1-ml sample (per plate) containing between 50 and 250 PFU. Plates were rocked every 10 min for 1 hr and then overlaid with Agarose (Wood, 1976) in TNM-FH containing 200 µg each of penicillin and streptomycin per ml and 5µg of Fungizone per ml. All assays were done in duplicate.

Peptide mapping. Protein samples of 1.5-3.0 mg of purified granulin or polyhedrin were digested with trypsin at a final substrate:enzyme ratio of 50:1 in 0.25 M ammonium bicarbonate (pH 8.6) at 38° for 12 hr. The hydrolysate was applied on Whatman 3MM filter paper and the peptides resolved in two dimensions by high voltage electrophoresis (Summers and Smith, 1975) (Brown and Hartly, 1966) as reviewed and outlined by Savant Instruments, Inc. (1971), "High Voltage Electrophoresis Guide". A third dimension of the neutral peptides was run using ascending chromatography with butanol:acetic acid:water (4:1:5) for 18 hr at room temperature. Positions of the hydrolysates were detected with 1.0% ninhydrin-0.1% cadmium acetate.

Amino acid analysis. Samples of 0.3 mg granulin or polyhedrin were hydrolized in a 6 N HCl at 105° for 24,48, and 72 hr. The hydrolysates were analyzed in a Beckman Model 120C automatic amino acid analyzer. The amounts of serine and threonine were obtained by extrapolating to time zero. Tryptophan content was based on adsorbance at 288 and 280 nm of protein dissolved in 6 M guanidine hydrochloride. All data presented are the mean values obtained from one analysis at 24 and 72 hr and three analyses at 48 hr.

Preparation of Antigens

Polyhedrins. Highly purified polyhedrin was isolated from the larval polyhedra of Autographa californica NPV (AcMNPV) by the method of Summers and Smith (1976) using preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The 30,000 molecular weight polyhedrin band was extracted from the gels and was analyzed for purity and stability by analytical SDS-PAGE.

Enveloped nucleocapsids from occlusions (LOVAL). Purified enveloped nucleocapsid preparations described as LOVAL (LOVAL: Larvae-derived Occluded Viruses which have been Alkali-Liberated, Volkman et al., 1976) were isolated from purified occluded virus using the method of Summers and Volkman (1976).

Enveloped nucleocapsids (NOV). AcMNPV EV (Plasma-Membrane Budded NonOccluded Virus) was purified from infected TN-368-10 (Volkman and Summers, 1976) culture medium by the method described previously (Summers and Volkman, 1976). This form of the virus has not been occluded or exposed to alkali. The physical and chemical properties of all viruses

and viral proteins utilized in this study have been characterized by SDS-PAGE, and the results of these comprehensive studies are to be published in a separate report (Summers and Smith, unpubl.). The stability and structural integrity of all preparations utilized in this study were routinely evaluated and documented as stable and reproducible.

Preparation of Antisera

Preimmune sera were obtained and rabbits were immunized essentially as described by Volkman et al. (1976) with the following purified antigens: AcMNPV LOVAL, AcMNPV EV, and AcMNPV polyhedrin.

Preparation of Purified Antibody (IgG)

Immunoglobin fractions were separated from antisera using two cycles of precipitation with 33% saturated ammonium sulfate. The precipitates were collected by centrifugation at 10.000 rpm for 15 min, and pellets were dissolved in and dialyzed against 0.0175 M phosphate buffer, pH 6.3. IgG was isolated by the method of Levy and Sober (1960).

^{125}I Labeling of IgG. Ig G was labeled with carrier-free Na^{125}I (New England Nuclear) by a modification of the chloramine-T method described by Greenwood et al. (1963). [^{125}I]IgG was separated from free ^{125}I using a Sephadex G-50 column (0.7 x 20 cm). [^{125}I]IgG was eluted and stored in 0.05 M phosphate buffer, pH 7.5, containing 1% bovine serum albumin (BSA) and 0.01% sodium azide. Specific activities of 3-5 x 10^6 cmp/µg of the protein were routinely obtained. Using 5% trichloroacetic acid (TCA) precipitation, more than 97% of the radioactivity was precipitable.

Microtiter Solid-Phase Immunoradiometric Assay (Micro-SPIRA)

Micro-SPIRA was carried out using a modification of the procedure of Purcell et al. (1973). The polyvinyl microtiter plate wells (Limbro Chemical Co., Inc.) were coated with 100 µl of antiserum which had been diluted 1:10 with phosphate-buffered saline, pH 7.3, containing 0.01% sodium azide (PBS). The microtiter plates were then incubated at 4°C for 4 hr to allow adsorption to the surfaces of the wells. After aspirating the fluid, the wells were washed twice with 200 µl of PBS. Two hundred microliters of 2% BSA solution (0.05 M phosphate buffer, pH 7.5, 2% BSA, and 0.01% sodium azide) were then added to each well to adsorb any unreacted sites. After incubation at 4°C overnight, the solution in each well was aspirated, and 30 µl of the appropriate dilution for the virus or polyhedrin was added. The microtiter plates were incubated at 4°C overnight (19-24 hr). The antigen solutions were aspirated, and each well was washed five times with 200 µl of the 2% BSA solution. Fifty microliters of [^{125}I]IgG in PBS (30-40 ng, 1 x 10^5 cpm) was added to each well and was incubated at 37°C for 4 hr. The wells were aspirated and washed five times with 200 µl of the 2% BSA solution. Individual wells were then cut from the microtiter plates, and the bound [^{125}I]IgG was determined in a Searle Model 1195 gamma spectrophotometer.

Inhibition Assay

Microtiter wells were coated with 1:10 dilutions of AcMNPV LOVAL antiserum and were incubated for 4 hr at 4°C. The wells were then aspirated, and nonreacted sites were coated with the 2% BSA solution overnight. The BSA solution was aspirated, and 100 µg of AcMNPV LOVAL in PBS was added to each well and was incubated at 4°C overnight. The wells were washed three times with PBS. Fifty microliters of the appropriate antiserum or IgG was delivered to each well and was incubated at 37°C for 4 hr. After aspiration of the antiserum, each well was rinsed five times with the 2% BSA solution, and 50 µl of [^{125}I]IgG was added. Following incubation at 37°C for 4 hr the wells were aspirated and washed five times with 2% BSA solution. Individual wells were then cut, and the bound radioactivity was counted as previously described.

Virus neutralization. Dilutions of antisera were made in Grace's medium, pH 7.5, with 0.5% bovine serum albumin and antibiotics. Virus samples were diluted in TNM-FH such that 0.1 ml of a 1:2 dilution would contain 100 to 200 pfu (Habel, 1969). Equal volumes (0.3 ml) of the prediluted antisera and the prediluted virus were mixed and allowed to incubate at either room temperature (28°C) or 37°C for 1 hr. Samples (0.1 ml) were then plated to determine the remaining pfu by the plaque assay. For some experiments sera were heated to 56°C for 30 min to remove any heat-labile nonspecific inhibitors or enhancers or virus infection.

Adsorption of antisera. Fifty microliters of undiluted anti-AcMNPV EV was added to 1 ml of 240 µg of AcMNPV LOVAL in 0.01 M Tris (pH 7.8) -0.01 M EDTA. This mixture was incubated at 37°C for 2 hr with occassional vortexing and then incubated again at 4°C for 72 hr. The suspension was then centrifuged at 100,000 x g and 5°C for 1 hr to remove the LOVAL. The supernatant fluid was carefully removed and used as a 1:20 dilution of adsorbed antiserum. When this serum was tested for residual LOVAL infectious activity, none was found.

Competition Radioimmunoassay (RIA). For RIA polyhedrins and granulins were purified by modifications of published procedures (Summers and Smith, 1976, 1978). The inclusion (polyhedra) associated protease was inactivitated by $HgCl_2$ and heat treatment (Summers and Smith, 1978) and polyhedrins and granulins obtained by electrophoresis in preparative vertical gel slabs in the presence of 0.1% SDS. Following electrophoresis the portion of the gel slab containing the polyhedrin or granulin was removed and extracted as described previously (Summers and Smith, 1975, 1976). Protein solutions were then stored at -90°C, and analyzed for stability and purity by analytical polyacrylamide vertical slab gel electrophoresis in the presence of 0.1% SDS (SDS-PAGE) (Laemmli, 1970) as modified for baculovirus proteins (Summers and Smith, 1978). For autoradiography gel slabs were dried onto filter paper then exposed to Kodak x-ray film for 48 hours.

AcMNPV polyhedrin and TnGV granulin were labelled with carrier free Na^{125}I (New England Nuclear) by the chloramine - T method (Greenwood et al., 1963). ^{125}I-protein was separated from free ^{125}Iodine using a P-60

(Bio-Rad) column. The labelled protein was stored in 0.01 M Tris buffer, pH 7.8, containing 2% bovine serum albumin (BSA) and 0.01% sodium azide (NaN_3) at -90°C, and used within two weeks since a progressive loss in immunoreactivity occurred upon extended storage at -90°C, storage at 4°C, and labelling of greater than 20 Ci/µg.

Cell extracts of infected or uninfected TN368-10 cell were prepared for RIA in the following manner. Log-phase cells in 25 ml plastic T-flasks were inoculated in a total volume of 1.0 ml with AcMNPV extracellular virus at a multiplicity of infection (MOI) of 10 to 20. After 1 hour adsorption, 4 ml of TNM-FH medium plus antibiotics were added. Mock infected cells received 1.0 ml of TNM-FH medium. Following adsorption, cells were washed free of inoculum by pelleting at 300 g for 15 min, and washed twice with 5.0 ml of TNM-FH media before resuspension in 5 ml of medium. At designated times post inoculation, cells were dispersed into a homogenous suspension by gentle shaking and pipeting, and samples removed for analysis. Cells were counted, adjusted to the appropriate concentration with medium, and pelleted by centrifugation at 300 g for 15 minutes. The cells were disrupted by suspension in 0.5 ml of 0.1 M Na_2CO_3 buffer, pH 10.9, containing 0.17 M NaCl, 0.01 M EDTA. Noindet-P-40 was added to a final concentration of 0.5%; the cellular suspension was allowed to set for 30 minutes and twice sonicated (30 seconds each at 4°C). The lysate was then centrifuged at 300 g for 15 minutes to remove any insoluble cell debris, and the supernatant carefully removed for assay of polyhedrin by RIA.

Immunodiffusion. Immunodiffusion tests were conducted by modification of the double diffusion method Ouchterlony (1958).

A modification of the micro-solidphase radioimmunoassay described by Purcell et al. (1973) was used for competition RIA. Two hundred µl of 0.05 M Na_2CO_3 - $NaHCO_3$ (pH 9.6) containing 5 µg of Protein A (Pharmacia Fine Chemicals, Inc.) were placed in flexible polyvinyl microtiter plate wells (Linbro Scientific Co.), incubated at room temperature for 2 hr and then overnight at 4°C. The solution was aspirated, the wells washed twice with distilled H_2O, and 200 µg of the appropriate dilution of purified antibody in 0.01 M Tris buffer solution added (pH 7.5; 0.1 M NaCl: 0.001 M EDTA; 0.01% sodium azide). The microtiter plates were incubated for 8.0 hr at 4°C. The wells were aspirated, washed twice with the Tris buffer and 10,000 cpm of ^{125}I polyhedrin or granulin (150 µl) in Tris-BSA buffer were added. The plate was incubated overnight at 4°C, twice washed with Tris-BSA buffer solution, and subsequently washed three times with Tris buffer. The microtiter wells were cut from the plate, and assayed for bound ^{125}I in an automatic gama spectrophotometer (Searle Model 1195).

Competitive inhibition assays were carried out in the same manner except the dilution of antibody was used which bound 50% of the homologous labelled polyhedrin or granulin. Competing proteins were diluted in the Tris-BSA solution so that each well received 150 µl of the polyhedrin or granulin solution. Following 24 hr of incubation at 4°C, 10,000 CMP of ^{125}I-polyhedrin or granulin (0.01 ml) was added and incubated overnight at 4°C. The wells were then washed, cut from the microtiter plates and counted.

For RIA detection of polyhedrin infected and non-infected TN368-10 cells, cell lysates were diluted in a 0.01 M Tris-HCl solution (pH 7.5; containing 0.01 M NaCl; 0.001 M EDTA; 0.05% NP-40; 0.01% sodium azide). Standard cell lysates with known polyhedrin concentrations were prepared by the addition of serial dilutions of purified polyhedrin ranging from 10 pg to 10 µg.

Immunoperoxidose Assay

Antisera. Rabbits (from which pre-immune sera were taken) were injected as previously described with highly purified AcMNPV larvae-derived occluded virions which had been alkali-liberated (LOVAL; Volkman et al.. 1976), or alternatively, with highly purified AcMNPV polyhedrin (Summers and Smith, 1976). Antiserum to purified AcMNPV AcMNPV EV was also tested (Summers and Volkman, 1976; Volkman et al., 1976). All antisera were heat-inactivated at 56°C for 30 min before use. Peroxidase-conjugated anti-rabbit IgG goat serum which contained 14 mg gamma globulin conjugated with 2.9 mg peroxidase per ml of solution was purchased from the Research Division, Miles Laboratories, Elkhart, Indiana. The antisera used in this study were shown to be specific for the homologous antigen(s) by a micro-solid-phase immunoradiometric assay, as they did not significantly cross-react with heterologous EV, LOVAL, or polyhedrin at concentrations less than 200 ng/ml of each antigen (Ohba et al., 1977)

Treatment of fixation of cells. Log phase cells were infected with AcMNPV EV at a multiplicity of infection (MOI) of not less than 5 or greater than 10 (Volkman and Summers, 1976). For time course studies, 10^7 cells of each subline, TN-368-10 and TN-368-13, were inoculated with EV at a MOI of 5. Other insect cell lines were infected at a MOI of 10. After a 1 hr adsorption period, the cells were pelleted by centrifugation at 300 g for 5 min and resuspended gently in complete medium. At this time and at regular intervals thereafter, samples of 10^6 cells were removed, pelleted by centrifugation at 300 g for 5 min, and rinsed twice in TNM-FH or TC100 containing 10% fetal calf serum. The cells were resuspended in a small amount of the appropriate serum-less medium by pipetting and small drops of each of the samples placed on acid-cleaned glass slides. The cells were allowed to attach to the slides for 20 min, then rinsed twice by gentle dipping in 0.01 M-phosphate buffered saline solution (PBS, pH 7.2). After washing, three different fixing procedures were compared: (1) the cells on the slide were air-dried for 20 min at room temperature, fixed in 1.5% paraformaldehyde in 0.067 M-potassium phosphate buffer, pH 7.4, for 20 min at room temperature, rinsed three times with PBS and air-dried again; (2) procedure 1 was used without the initial air-drying step; (3) the cells were fixed in acetone for 10 min at room temperature.

The other invertebrate cell lines were processed using the same routine procedures and reagents employed with TN-368-10 and TN-368-13 cells.

Immunoenzymic staining procedure. The procedure used was basically that obtained from Dr. Heather Mayor (personal communication; Baylor

Medical Center, Houston, Texas) with a few modifications. After fixation, the cells were covered with one or two drops of rabbit antiserum diluted 1:100 to 1:200 in PBS, and were incubated at 37°C for 1 hr. The proper dilution of antiserum was determined experimentally by selecting the dilution which gave the least nonspecific but the most intense specific (positive) reaction. Cells were rinsed extensively in five consecutive changes of PBS before adding one or two drops of a 1:100 dilution of peroxidase conjugated goat anti-rabbit IgG. After a 1 hr incubation at 37°C, the cells were rinsed extensively in five changes of PBS. The peroxidase substrate (made up freshly) was 0.4 mg/ml 3-3' diaminobenzidine (DAB) in 0.01% hydrogen peroxide (H_2O_2) and 0.1 M-Tris, pH 7.6. The stock bottle of powdered DAB was kept frozen in a desiccator and the H_2O_2 working dilution was made fresh each time from a 30% stock solution. The cells were covered with the substrate solution and were allowed to incubate for 10 min at room temperature in the dark. Following this treatment they were washed extensively in PBS, dehydrated and mounted with permount for microscopic examination. Dehydration was accomplished by immersing slides sequentially for 3 min in each of the following solutions: 25%, 50%, 75%, 90%, and 100% ethanol, and xylene.

A total of 400 cells in each of three replicate samples were scored to determine the percentage reacting with immunoperoxidase.

Tests for sensitivity and specificity of the assay were performed. The tests for specificity included the substitution of pre-immune sera for the enveloped nucleocapsid or polyhedrin antisera, and the use of non-infected cells. In tests for the appropriate concentrations of antisera and reagents, dilutions ranging from 1:10 to 1:400 were tested. Additional controls involved the omission of either virus antiserum, peroxidase conjugated antiserum, DAB or hydrogen peroxide.

Intracellular and Extracellular viral growth curves. For virus growth curves TN-368-10 cells were inoculated as described and assayed in triplicate. At the designated time, 1×10^6 cells were removed, pelleted by centrifugation at 300 g for 10 min, and the supernatant saved for extracellular virus tiration. The cells were twice resuspended in 5 ml of TNM-FH plus 0.1 mg/ml of gentamycin and 5 mg/ml of fungizone, and twice pelleted by centrifugation. After this, they were resuspended in 1.1 ml of the medium, pelleted again, and 0.1 ml of final supernatant tested for residual infectivity by the polyhedral plaque assay. The pelleted cells were resuspended in the remaining medium, and disrupted using a Sonifier Cell Disruptor (Heat Systems Ultrasonics, Inc.) at 4°C for 1 min at setting 3, and then for an additional 0.5 min at setting 5. The effects of sonication on virus infectivity were tested by adding known titres of EV to TN-368 cells and disrupting the cells as above. The procedure routinely destroyed 30% of virus infectivity and all values reported in these studies were corrected accordingly. The cell debris was pelleted at 2000 g for 10 min and the infectivity of the supernatant solution was determined by polyhedral plaque assay.

Plaque-purification for restriction enzyme studies. Three forms of AcMNPV were used to produce the plaques from which each isolate was purified. These were AcMNPV extracellular (E), AcMNPV single (S), and AcMNPV multiple (M) (Summers and Smith, 1978). A total of 15 viral

plaque isolates, five each for E, S, and M, were plaque-purified 3 times and then grown to 20 ml of infectious tissue culture supernatant.

The method used for plaque-purifying wild isolates of AcMNPV was a modification of the polyhedral plaque assay of Hink and Vail (1973), as additionally modified by Volkman et al., (1976). Log-phase TN-368 cells were seeded into 35-mm Corning tissue culture dishes at a density of 7.0×10^5 cells/plate in 2.0 ml of medium without fetal calf serum. After the cells attached the medium was removed, and 0.1 ml of virus dilution was added. The plates were rocked every 15 min for 1 hr, after which the inoculum was removed and the plates were overlaid with 1.5% agarose (Wood, 1977). In the case of singles (S) and multiples (M), the overlay contained a 1:20 dilution of AcMNPV antiserum.

Individual plaques were selected from plates showing no more than five widely separated plaques. Plaques were removed with a 1-cc disposable tuberculin syringe and 22 gauge needle using a stereoscope to visualize the plaque. The plaque was diluted and replated on uninfected TN-368-10 cells in the same manner described above. Each isolate was plaque-purified 3 times in this manner before preparing sufficient quantities of virus for DNA studies.

Purification of virus and viral DNA for restriction analysis. The polyhedra from infected TN-368-10 cells were purified by sonication (10 sec at a setting of 4 with a model W375 Sonicator equipped with a micro tip, Ultrasonics, Inc., Plainview, N.Y.). The sonicated suspension was centrifuged in 40-63% (wt/wt) sucrose gradients, and polyhedra separated from cellular materials as described by Summers and Smith (1978).

AcMNPV extracellular virus was purified as described by Summers and Volkman (1976). DNA was purified using a modification of the procedures of Summers and Anderson (1973) from purified virus (Summers and Smith, 1978). Each virus preparation was prepared at a concentration of 1 mg protein/ml in 0.1 M Tris, 0.01 M EDTA, pH 7.5, then made 2% with respect to sodium lauryl sarcosinate and incubated at 37°C for 15 min. Pronase was added to a final concentration of 2 mg/ml and incubated for 4 hr at 37°C. Each was extracted 3 times with buffer-saturated phenol and the aqueous layers recovered and layered on CsCl gradients with a mean density of 1.50 g/ml containing 200 µg/ml ehtidium bromide. Centrifugation was conducted for 36 hr at 35,000 rpm using an SW-41 rotor. After centrifugation, the DNA was removed by piercing the bottom of the tubes and collecting the UV-visible fractions. The two bands which contained covalently closed DNA (1.58 g/ml) or linear and relaxed circular DNA (91.54 g/ml) were collected separately, extracted 3 times with isoamyl alcohol, and dialyzed extensively against 1.0 M Tris, 0.1 M EDTA, pH 7.5.

Restriction endonuclease digestion. Purified viral DNA was digested with EcoR-1 or Hind III restriction enzymes (Boehringer Mannheim, Indianapolis, Ind.) EcoR-1 digestion mixtures (total of 20 µl) contained 2 µg DNA, 0.05 M NaCl, 0.01 M $MgCl_2$, 0.1 M Tris-HCl, pH 7.5, and 10-15 units of enzyme were incubated for 1 hr at 37°. Hind III enzyme digests (total of 20 µl) contained 2 µg DNA, 0.05 M NaCl, 0.01 M $MgCl_2$, 0.014 M dithiothreitol, 0.01 M Tris-HCl, pH 7.6, and 10-15 units of enzyme and were incubated for 1 hr at 37°. Enzyme activity was stopped by adding 2.0 µl of 0.1 M EDTA, 50% glycerol, and 0.5% brom-phenol blue. The samples

were heated for 15 min at 60° then quenched on ice before layering on the gel slab.

Lambda DNA (Miles Laboratories, Inc., Elkart, Ind.) and adenovirus-2 DNA (gift from Dr. Steve Bachenheimer; University of North Carolina, Chapel Hill, N.C.) were digested with EcoR-1 as described above.

The restriction endonuclease (Sma-1) isolated from Serratia marcescens (Boehringer, Mannheim) was incubated with 2 µg lambda DNA, 0.015 M Tris-HCl, pH 8.5, and 10 units of enzyme added for 30 min at 37°. The sample was prepared for electrophoresis as described above.

Agarose electrophoresis. Horizontal 0.5, 0.75, and 1% agarose (Bio-Rad Laboratories, Richmond, Calif.) slab gels were prepared on a LKB model 2117 Multiphore electrophoresis apparatus. Analytical gels (3 x 150 x 250 mm) were prepared with 4 x 0.75 or 8 x 0.75 mm wells. Agarose was dissolved in 40 mM Tris, pH 7.8, containing 20 mM sodium acetate, 1 mM EDTA, and 0.5 µg/ml ethidium bromide. This buffer was also used as the electrode buffer. Gels were prerun for 30 min before samples were applied.

The DNA was electrophoresed at a constant voltage of 50 V and at 15° for 18 to 25 hr. DNA fragments in discrete bands were visualized under long wave UV illumination using a transilluminator (UV Products, San Gabriel, Calif.). Photographs were taked on Kodak Pan Contrast Process film using a Wratten No. 15 filter. The agarose gel slabs were dried under vacuum and the ^{32}P-labelled fragments detected by exposing the dried gel to Kodak NS-54T x-ray film for 2 days. Autoradiograms were scanned with an E-C model scanning densitometer equipped with a Perkin-Elmer M-2 calculating integrator.

EcoR-1 restriction endonuclease and molecular weights virus DNAs. Viral DNAs from seven nuclear polyhedrosis viruses and two granulosis viruses were cleaved with EcoR-1 and electrophoresed in 0.75% agarose. The molar ratios of fragment bands were determined by plotting the migration versus the log of the integral area as determined by scanning autoradiograms or photographic negatives of UV flourescent bands with a scanning densitometer equipped with a digital integrator. Migration of EcoR-1 fragments of lambda phage, adenovirus-2 DNA, and the Sma-1 fragments of lambda phage were plotted versus the log molecular weight to provide a standard curve. Molecular weights of EcoR-1 fragments of lambda and adenovirus-2 were as reported by Helling et al. (1974 and Pettersson et al. (1973), respectively, and the Sma-1 fragment molecular weights correspond to values reported by McParland et al. (1976). Mean baculovirus DNA fragment molecular weights were determined from electrophoresis in three agarose gels.

SECTION 5

Results and Discussion

A. Granulins and Polyhedrins:

We have reported procedures and techniques which are able to differentiate and identify the structural properties of baculovirus polyhedrins and granulins (Summers, 1975; Summers and Smith, 1975, 1976, 1978). Two-dimensional high voltage peptide mapping has been a most useful technique for providing a diagnostic fingerprint for identity which can be supported by additional data derived from SDS polyacrylamide gel electrophoresis, amino acid analysis, and amino-terminal determination (Maruniak and Summers, 1978). These comparisons are essential as a continuing basis for investigating the structural properties of polyhedrins relative to the sensitivity and specificity of serological techniques. Since cross-reactions measured by various serological techniques have been reported for polyhedrins (Bell and Orlob, 1977; Croizier and Meynadier, 1973, 1975; Guelpa et al., 1977; Harrap et al., 1977), a comparison of primary structure peptide mapping of these proteins will provide information to help predict and explain these cross-reactions as well as in establishing the identity of individual granulins and polyhedrins.

1. Molecular Weights. The granulins and polyhedrins of eight baculoviruses were compared (Figure 1). All polyhedrins and granulins exhibited a single major component with mobility between chymotrypsinogen (25,000) and deoxyribonuclease (31,000). The molecular weights of each monomer subunit were estimated to the nearest 500 daltons and were as follows: AcMNPV, 30,000; RoMNPV, 30,000; AgMNPV, 29,000; HaMNPV, 28,000; TnSNPV, 31,000; HaSNPV, 27,000; TnGV, 25,000; and SfGV, 26,000.

2. Two-dimensional, high-voltage electrophoresis (HVE). Figures 2-3 show the results of HVE of granulins and polyhedrins. Unfortunately, the neutral peptides migrating on the 0/n line are not highly resolved using this technique. A third dimension of ascending chromatography using butanol:acetic acid:water (4:1:5) allows resolution of those peptides. Visual inspection of each of the maps shows that there are similarities among all proteins studied.

Tables I-III provide a more detailed analysis of the extent of similarity and disimilarity. Degrees of similarity may be evaluated by comparing peptides which are similar by relative migration to the total number of peptides among those proteins being compared.

Certain peptides of the basic, acidic, and neutral peptides were found common in all of the granulins and polyhedrins. For example, the basic peptide 8, of the 1/2 diagonal (Table I) is found in every protein. Other widely distributed basic peptides are 3 of the 1/3 diagonal, basic peptides 4, 5, 6, and 9 of the 1/2 diagonal, and basic peptide 4 of the 2/3 diagonal. The most prevalent acidic peptide common to all proteins

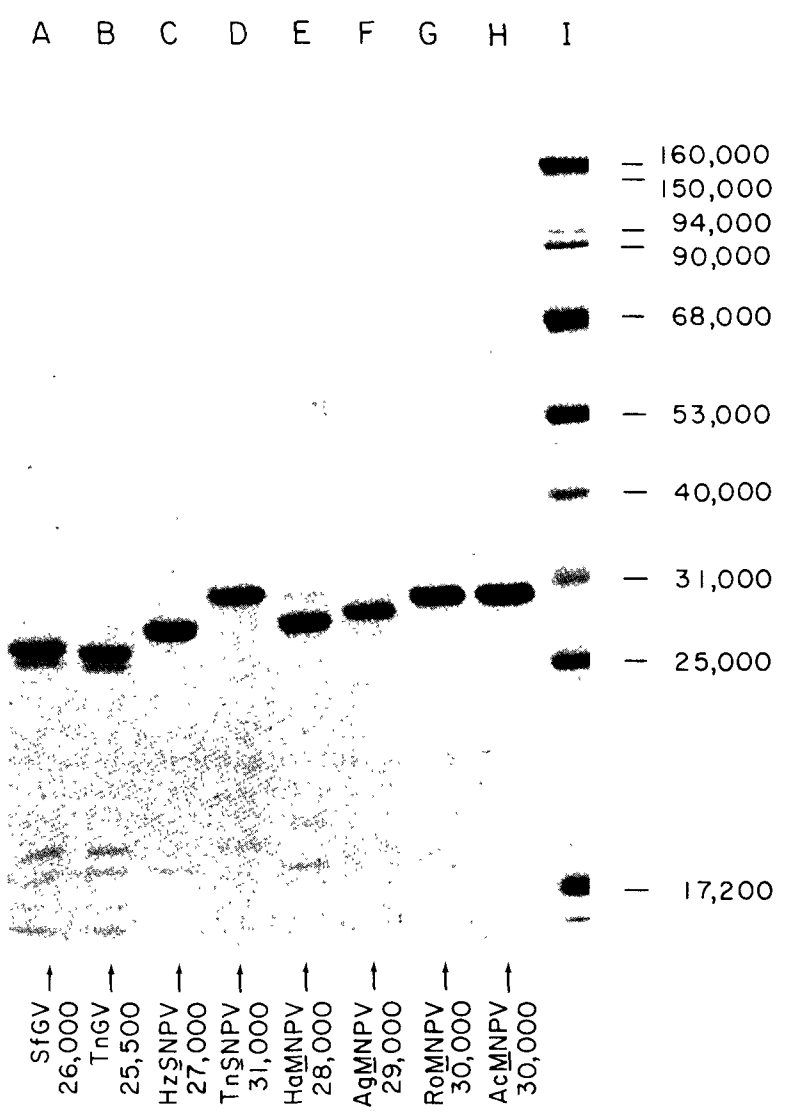

Figure 1. Granulins and polyhedrins were purified as described in Materials and Methods and disrupted in 2% SDS and 5% mercaptoethanol, and 1.5 µg of each was electrophoresed in 11% polyacrylamide slab gels in the presence of 0.1% SDS. SfGV and TnGV granulin and HzSNPV, TnSNPV, HaMNPV, AgMNPV, RoMNPV, and AcMNPV polyhedrin are shown in A-H, respectively. The molecular weight of each polyhedrin or granulin is given below each sample. The standard proteins are shown in I and molecular weights are indicated on the right.

TABLE I: Basic Peptides

Diagonal	¼		⅓						½												⅔				
Peptide No.	1	2	1	2	3	4	5	6	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5
Ag*M* NPV		+	+	+	+	+	+	+			+		+		+	+		+			+	+	+	+	
Ha*M* NPV	+		+						+	+	+	+		+		+	+	+			+		+	+	+
Hz*S* NPV					+							+	+	+		+	+	+	+	+				+	+
TnGV	+	+	+		+					+	+	+	+	+	+	+	+								
SfGV	+		+		+					+	+	+	+	+	+	+							+	+	+
Tn*S* NPV			+		+						+	+	+	+		+	+	+	+		+		+	+	
*Ro*MNPV				+	+					+	+	+	+	+		+	+	+			+		+	+	
Ac*M* NPV				+	+				+	+	+		+		+	+	+				+	+	+	+	

[a] Composite of tryptic peptides located by two-dimensional high-voltage electrophoresis and descending chromatography derived from AgMNPV, Anticarsa gemmatalis; HaMNPV, Heliothis armigera; HzSNPV, Heliothis zea; TnGV (Summers and Smith, 1976), Trichoplusia ni; SfGV (Summers and Smith, 1976), Spodoptera frugiperda; TnSNPV (Summers and Smith, 1976), Trichoplusia ni; RoMNPV (Summers and Smith, 1976), Rachiplusia ou; AcMNPV (Summers and Smith, 1976), Autographa californica.

TABLE II: Acidic Peptides

Diagonal	$\lvert -n/n \rvert > 1$								$\lvert -n/n \rvert < 1$										
Peptide No.	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	9	10	11
Ag*M* NPV	+	+		+					+		+			+	+	+	+		
Ha*M* NPV	+		+				+	+		+	+		+	+					+
Hz*S* NPV		+	+				+		+			+	+	+				+	
TnGV	+				+				+	+	+			+					
SfGV	+	+							+	+			+						
Tn*S* NPV	+	+	+	+	+					+			+	+					
Ro*M* NPV	+	+	+		+								+						
Ac*M* NPV	+	+	+				+		+			+		+					

[a] See Table I, Footnote a.

TABLE III:

NEUTRAL PEPTIDES[a]

	A		B							C									D					E			F			G
Peptide No.	1	2	1	2	3	4	5	6	7	1	2	3	3a	4	4a	5	5a	6	1	1a	2	2a	3	1	1a	2	1	2	3	1
Ag*M* NPV	+		+	+			+			+				+	+				+							+			+		
Ha*M* NPV		+			+							+	+	+				+				+		+	+	+		+			
Hz*S* NPV	+	+		+		+	+							+	+	+	+			+	+	+	+			+					
TnGV	+		+							+	+			+		+			+							+	+	+		+
SfGV	+		+			+				+	+							+	+							+	+	+		+
Tn*S* NPV				+	+		+	+		+	+							+	+					+		+	+	+		+
Ro*M* NPV			+			+	+		+	+	+					+		+	+					+			+	+		+
Ac*M* NPV				+						+	+	+							+		+		+	+		+	+	+		+

[a] See Table I, Footnote a.

are C1, D1, E2, and F2. HaMNPV, HzSNPV, and AgMNPV polyhedrins appear to be most related to previously characterized granulins and polyhedrins on the 1/2 diagonal (Table I) of the basic peptides, indicating that the primary sequence of this region of these proteins has been the most stable and conserved through evolution.

Comparisons of the polyhedrins and granulins by amino-terminal analysis reveals three different groupings: The amino-terminus for AcMNPV, RoMNPV, and AgMNPV are proline; for HzSNPV, HaMNPV, and TnSNPV, methionine; and TnGV and SfGV, glycine. It is interesting to note that the amino acid sequence of polyhedrin from Bombyx mori NPV (Serebryani, et. al., 1977) shows the amino-terminal amino acid to be proline which we have determined as the amino-terminus for AcMNPV, RoMNPV, and AgMNPV.

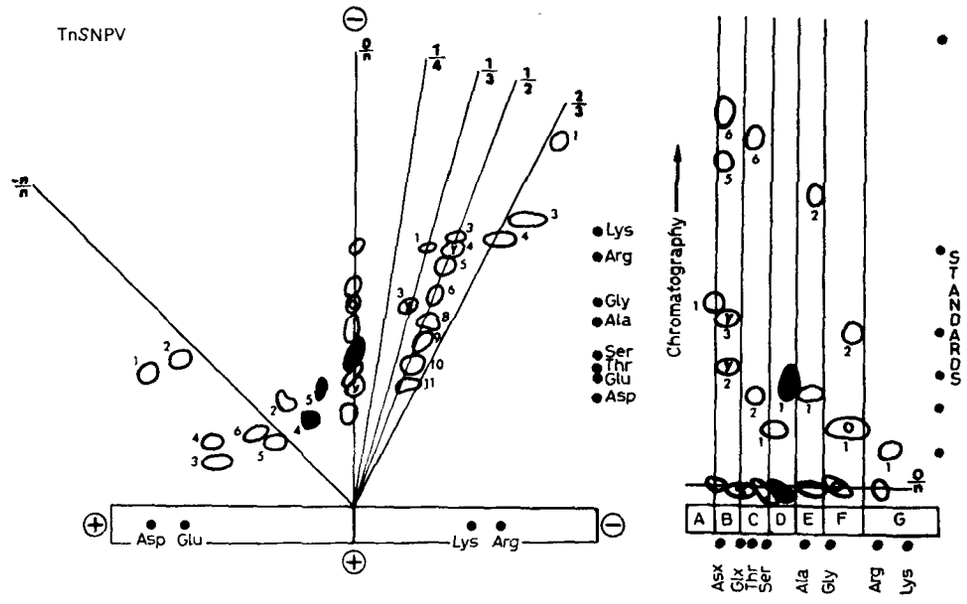

Figure 2-3. Two-dimensional high-voltage electrophoresis of a tryptic digest of granulins and polyhedrins. Electrophoresis was carried out on Whatman 3 MM filter paper at pH 6.5 (10% puridine - 4% acetic acid) for the first dimension (3 kV, 35 min) and at pH 1.9 (8% acetic acid - 2% formic acid) for the second dimension (3 kV, 30 min). Chromatography of the neutral hydrolysates was run in butanol:acetic acid:water (4:1:5) for 18 h at room temperature, positions of hydrolysates were detected with 1% ninhydrin - 0.1% cadmium acetate. Dark spots indicate peptides which were UV-positive; y=yellow peptides; o=orange peptides; AcMNPV=Autographa californica NPV; HzSNPV=Heliothis zea nuclear polyhedrosis virus.

We have presented data on the primary structure of granulins and polyhedrins. This information has been useful to identify and differentiate the species specific granulins and polyhedrins. At the same time, information is provided that shows similarities among the virus proteins which can account for serological cross-reactions (to be evaluated later) that have been observed by this and other laboratories. As summarized in these studies, baculovirus granulins and polyhedrins demonstrate remarkable similarities in size, composition, and chemical properties. However, it is obvious from the results reported herein that for those viruses investigated, each granulin and/or polyhedrin is a specific protein in association with a given species of occluded virus. The proteins appear to be of a family or class with degree of relatedness in terms of similar primary structure. The degree of relatedness among the polyhedrins is not nearly as close as between granulins.

Serological studies to date (Mazzone, 1975; Norton and DiCapua, 1975) have shown cross-reactivity in antisera prepared against various granulins and polyhedrins when tested against heterologous granulins and polyhedrins, as well as when tested against purified virus preparations of the DNA baculoviruses. If the chemical relationships hold true for other viruses as observed in this study, the cross-reactivity of antigens and antisera is partially understood. However, it is difficult at this time to draw further conclusions about degrees of similarity among these proteins until more proteins are adequately characterized. It is now possible, however, to correlate serological studies on these proteins at the quantitative level with the degree of chemical similarity among polypeptides. The technique of two-dimensional HVE which arranges peptides along a series of lines, each line ascribed to two coordinates based upon the net charge on the peptide during HVE at 6.5 and 2.0, is an extremely effective technique for qualitative comparisons of these proteins. In many cases single amino acid differences can be detected in major peptides.

It is essential that alkaline protease activity be eliminated for comparative studies. The very similar structure of the SfGV and TnGV points to the need for very careful comparisons since it has been previously shown (Summers and Smith, 1975) that alkaline protease activity can induce considerable artifact showing significant differences in the migration of tryptic peptides from enzyme active versus nonactive preparations.

B. Structural Polypeptides of Enveloped Nucleocapsids and Nucleocapsids:

1. Virus Polypeptides

Figures 4 and 5 show the relative mobilities of the structural polypeptides for eight baculoviruses. Only enveloped single nucleocapsids were utilized for this initial comparison, and not multiples of nucleocapsids. The results confirm that baculoviruses are structurally complex. Although some bands exhibited similar mobility for different

viruses, for example, AcMNPV and RoMNPV appear closely related, each virus was unique when comparing mobility and the total number of bands resolved.

The molecular weights of enveloped nucleocapsid proteins (VP) and of polyhedrins and granulins were routinely determined by comparison with the relative mobilities of standard marker proteins. The molecular weights of the VPs ranged from 15,000 to 160,000 (Figures 4 and 5).

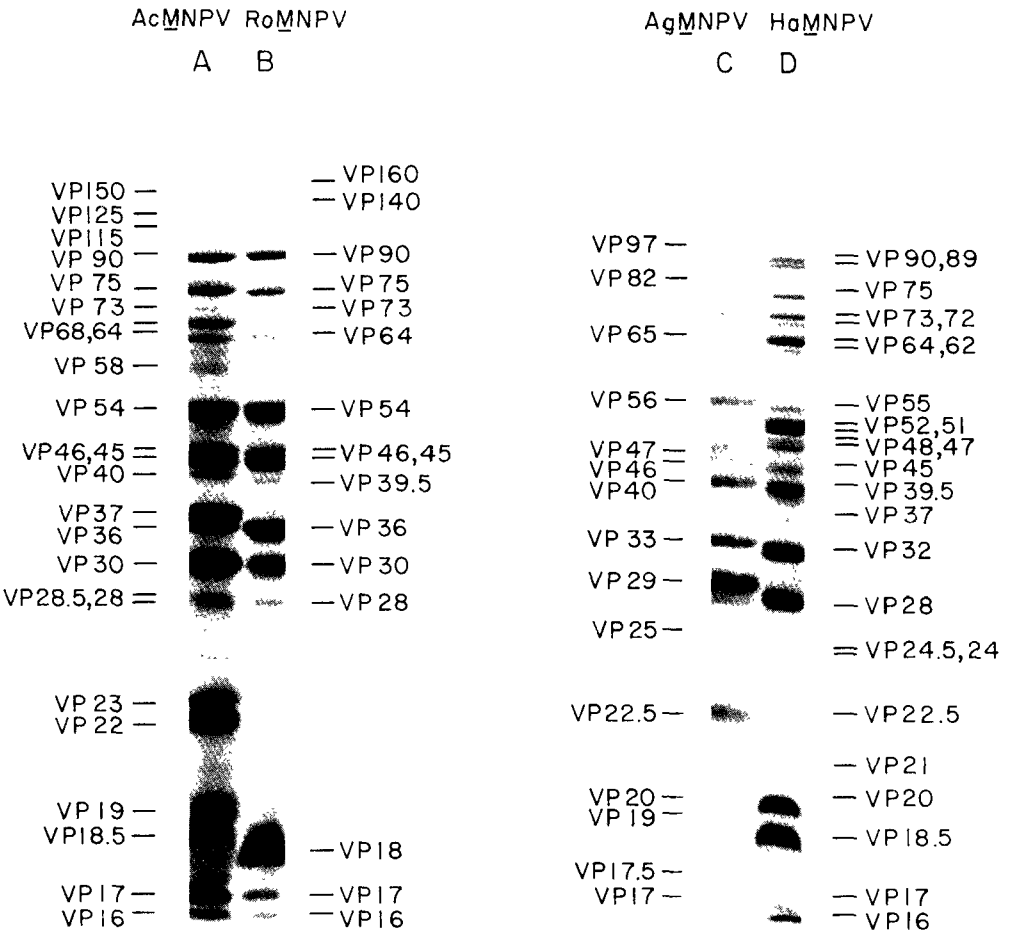

Figure 4. Polyacrylamide slab gel electrophoresis of enveloped nucleocapsid polypeptides. The viruses were purified by equilibrium banding in sucrose (1.17 to 1.25 g/ml) after being liberated from the polyhedra with 0.1 M Na_2CO_3, 0.01 M EDTA, 0.17 M NaCl, at 0 to 4° for 60 min. The band containing only one nucleocapsid per envelope (singles) was removed from the gradient and electrophoresed in 11% polyacrylamide as described in Materials and Methods. The virus polypeptides (VPs) of AcMNPV, RoMNPV, AgMNPV, and HaMNPV are shown in A, B, C, and D. The molecular weights of the virus polypeptides were estimated by comparison

of relative mobilities using the method of Weber and Osborn (1969). Each VP that was resolved is marked with the calculated molecular weight times 10^{-3}. The molecular weights of the standard protein markers are: RNA polymerase subunits, 160,000, 150,000, 90,000, and 40,000; phosphorylase a, 94,000; bovine serum albumin, 68,000; L-glutamic dehydrogenase, 53,000; DNA nuclease, 31,000; chymotrypsinogen, 25,000; and myoglobin, 17,200.

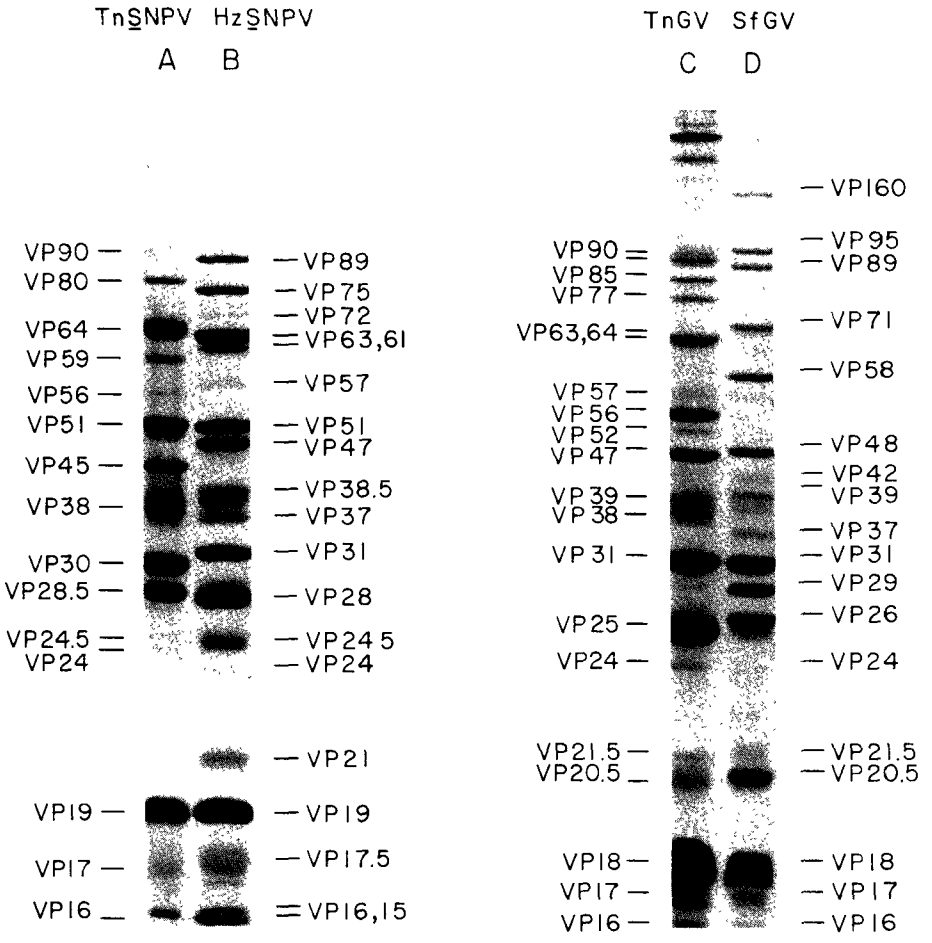

Figure 5. Baculovirus-enveloped nucleocapsids were prepared and electrophoresed as described in Fig. 4. The VPs of TnSNPV, HzSNPV, TnGV, and SfGV are shown in A, B, C, and D, respectively.

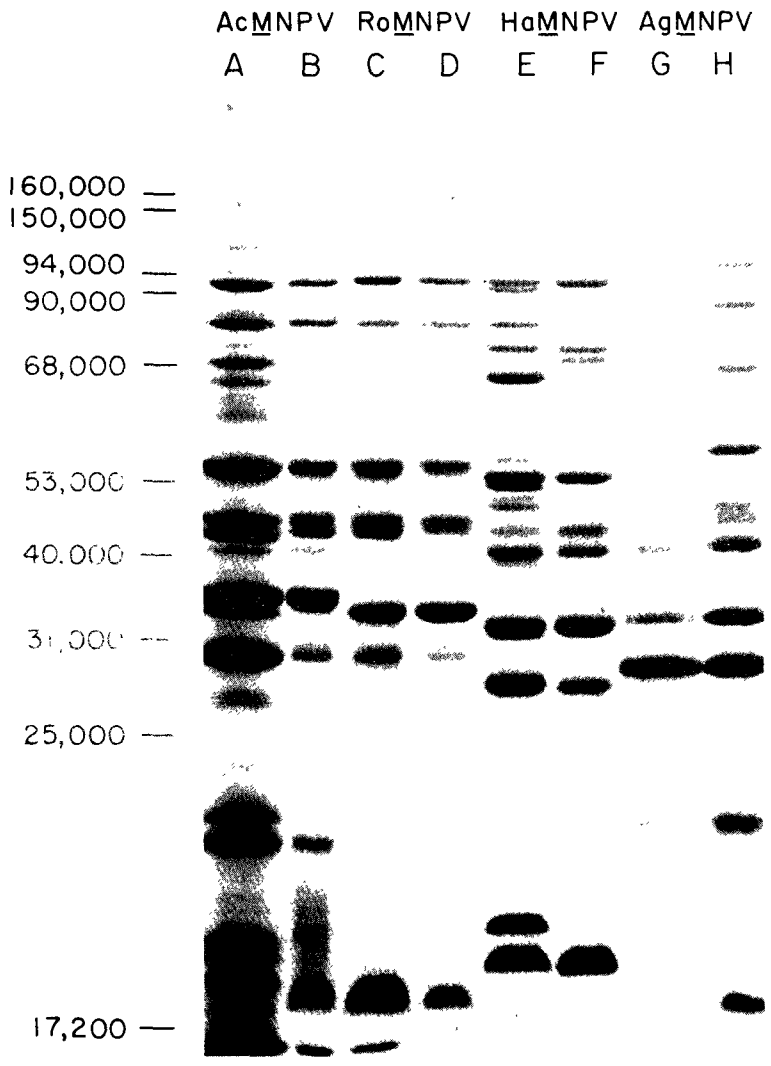

Figure 6. Comparison on an 11% polyacrylamide slab gel of polypeptides from enveloped single nucleocapsids and multiples. Each of the four MNPVs used in this study was liberated from the polyhedra and gel electrophoresis was conducted as described in Fig. 4, except that in addition to collecting the top virus band (singles), the remaining virus band (multiples) were also collected and analyzed. The VPs of singles and multiples, respectively, of AcMNPV are observed in A and B; RoMNPV, C and D; HaMNPV, E and F; and AgMNPV, G and H. The position of the molecular weight standards are indicated next to the VPs.

2. Polypeptide Composition of Enveloped Singles and Enveloped Multiples of Nucleocapsids

After centrifugation to quasiequilibrium in sucrose gradients, the least dense virus band (density of 1.20 to 1.21 g/ml) of enveloped single nucleocapsids from AcMNPV, RoMNPV, AgMNPV, and HaMNPV was separated from the multiples (usually seven to eight additional bands ranging in density from 1.22 to 1.25 g/ml) and was compared by polyacrylamide gel electrophoresis (Figure 6). The results show that both qualitative and quantitative differences exist between multiples and singles for each virus. Since comparisons of gel profiles were made by inspection, qualitative differences in protein composition will be emphasized. It is not desirable to elaborate upon the more subtle quantitative differences without a more accurate means of quantitation.

VP30, VP28, VP19, VP18.5, and VP16 of AcMNPV singles are observed to be in relatively smaller concentration when compared to multiples (Figure 6A and B). VP58, VP36, and VP23 are apparently absent or diminished in relative concentration to a level not detectable in the gels. The polypeptide composition of HaMNPV singles shows that VP64, VP47, VP28, and VP20 are present in increased amounts relative to the same protein in multiples (Figure 6E and F), whereas VP89, VP75, VP62, and VP51 are not observed in multiples. In contrast, there is more of HaMNPV VP45 in multiples as compared to singles. The polypeptides of RoMNPV, singles, VP90, VP54, VP46, VP45, VP30, VP18, and VP16, exist in greater amounts as compared to multiples; VP160 is consistently more prominent in multiples (Figures 6C and D). Quantitative differences are also observed for VP22.5 and VP17.5 in AgMNPV, however, the quantitative relationship between singles and multiples is different than those of the other viruses. The explanation for this is not apparent to us at this time.

3. Polypeptides of Baculovirus Capsids

AcMNPV capsids purified after the above treatment are composed of two major polypeptides, VP37 and VP18.5, and trace quantities of VP30.5 and VP30 (Figure 7B). RoMNPV capsids have several major polypeptides; VP61, VP17, VP18, VP30, and VP36, with detectable quantities of VP30.5 (Figure 7D). The presence of two polypeptides between VP30 and VP36 is not presently understood since these two proteins are not detected in intact virus preparations (Figures 4 and 6). The TnGV capsid is composed of two major polypeptides, VP31 and VP17 (Figure 7I), with trace quantities of VP29 and VP26. Each capsid preparation consistently contained several other minor polypeptides (most of which have been discussed) which could be components of the viral capsid. It is presently assumed that the differences between capsid and enveloped nucleocapsid protein composition shown in Figure 7 reveals that the envelope composition of baculoviruses is quite complex. For example, AcMNPV contains a total of approximately 18 bands (Figures 6B and 7A). Since there are only three capsid proteins, the envelope must contain a majority of the remaining. This relationship, although different for each virus studied, is consistent among all comparisons made (Figure 7).

It is easily seen that each of the baculoviruses has a unique

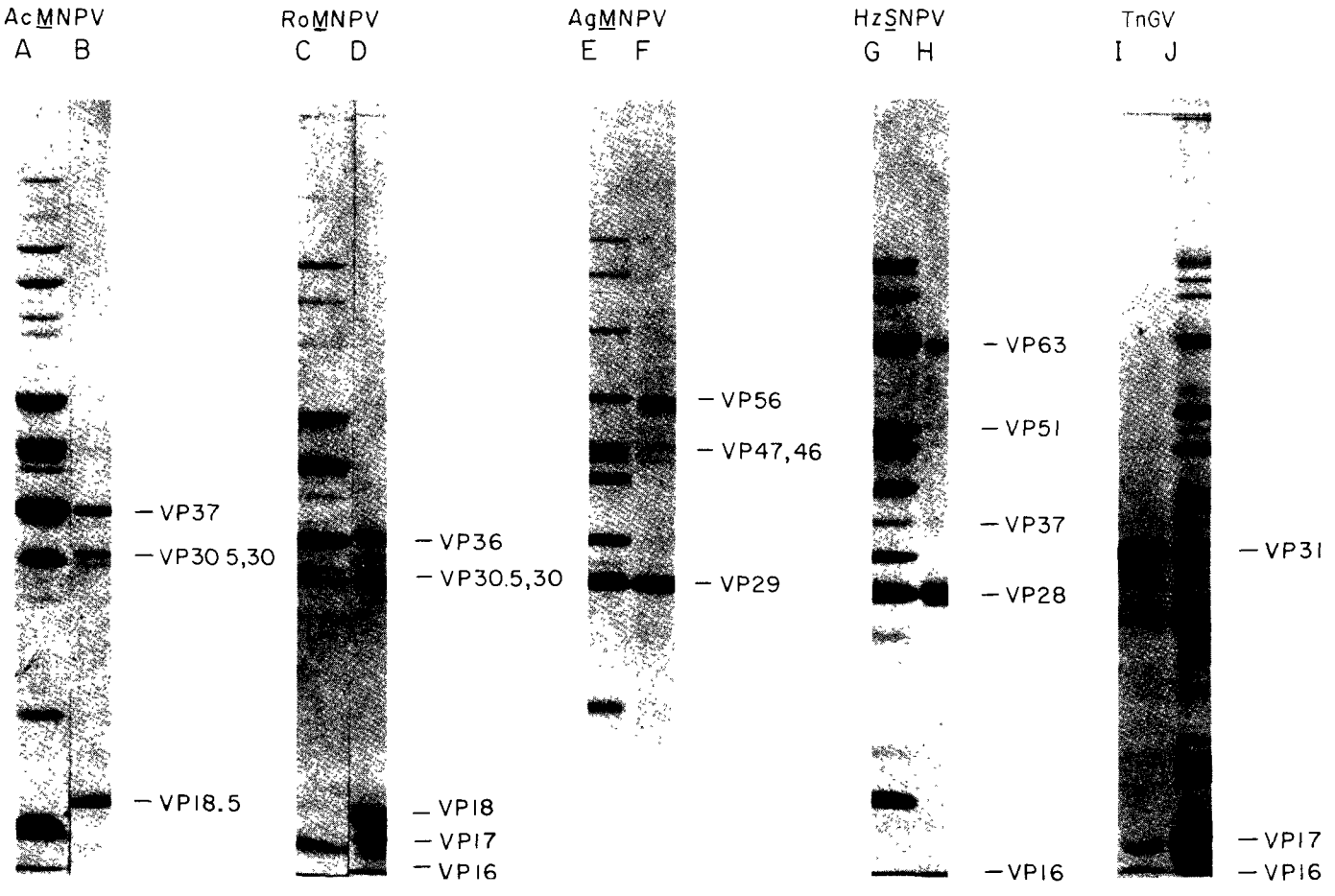

Figure 7. AcMNPV, RoMNPV, and TnGV were treated with 2% NP-40 in 0.01 M Tris (pH 7.8), 0.01 M EDTA for 18 hr at 37°, virus capsids (density of 1.33 g/ml) were recovered, and 5 µg of each was prepared for electrophoresis in 11% polyacrylamide slab gels. AcMNPV, RoMNPV, and TnGV capsid proteins are shown in B, D, and I, respectively. AgMNPV (F) was treated with 2% NP-40 in 0.01 M Tris (pH 7.8), 0.01 M EDTA, 1 M NaCl for 18 hr at 37°, then centrifuged and electrophoresed as above. HzSNPV (H) was treated with 2% NP-40, 0.01 M Tris (pH 7.8), 0.01 M EDTA, 1 M NaCl for 18 hr at 37°, then treated with mercaptoethanol and incubated for 1 hr at 70°. The capsid bands were removed from CsCl gradients and 5 µg was electrophoresed as described previously. Adjacent to each capsid preparation in a nontreated control of enveloped nucleocapsids that had been repurified on CsCl gradients.

protein composition. However, there is little that can be deduced from the structural approach presented herein using present information as to the relationships among baculoviruses in terms of strain, species, etc. The possible exceptions are AcMNPV and RoMNPV which have many proteins with similar electrophoretic mobilities. This does not imply that these proteins are identical, and additional biochemical and serological characterization of each protein will be necessary to establish the existence of structural similitaries. A comparison of AcMNPV and RoMNPV proteins from multiples in Figure 7 shows that two major proteins, VP37 (AcMNPV) and VP36 (RoMNPV), have different electrophoretic mobilities. Also, AcMNPV has at least three additional major proteins, VP22, VP23, and VP19, which are not observed in RoMNPV. Peptide mapping analysis of RoMNPV and AcMNPV polyhedrins has shown that only 40% of the tryptic peptides of the two proteins have similar mobility (Summers and Smith, 1976). However, Volkman et al. (1976) using comparative neutralization could not show differences by kinetic neutralization between RoMNPV and AcMNPV. The results of highly specific and sensitive serological assays for enveloped nucleocapsids utilizing double gel diffusion, and competition radioimmunoassay (Hoops and Summers, 1978, manuscript in preparation) and immunoradiometric assay (Ohba et al., 1977) also indicate a great deal of similarity between AcMNPV and RoMNPV. The apparent close relatedness of AcMNPV and RoMNPV may provide an interesting example for continued studies relating structural similarity of baculovirus strains to biological activity and specificity.

The quantitative and qualitative differences in virus polypeptides observed between singles and multiples in MNPVs may, in part, be explained by the ratio of envelope to nucleocapsid protein. The DNA:protein ratio is 0.20 for enveloped single nucleocapsids and 0.30 for AcMNPV multiples (Volkman et al., 1976). The significance of the difference in protein composition between singles and multiples is not clear to us. Whether the quantitative differences and the presence or absence of certain polypeptides may also be related to biological differences is also difficult to evaluate using present evidence. However, this study has prepared the basis for comparison with plaque purified viruses in order to reveal whether or not the polypeptide composition of multiples and singles is related to strain differences and/or is a genetically stable trait.

4. Structural Polypeptides of Extracellular Virus

The structural polypeptides of AcMNPV purified by alkali extraction from the polyhedra of infected insects were analyzed by polyacrylamide electrophoresis (Summers and Smith, 1978). A comparison of the proteins from polyhedral-derived virus with those of extracellular virus from cell culture medium (Fig. 8) revealed quantitative and qualitative differences in polypeptide composition. Extracellular virus has a much greater quantity of VP64, and several other minor polypeptides, VP70, VP60, and VP27 were not detected in singles or multiples.

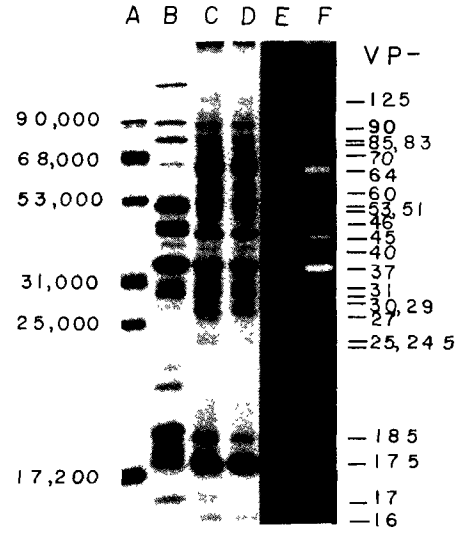

Figure 8. A comparison of AcMNPV from the polyhedra of infected TN-368-10 cells with extracellular virus by electrophoresis in 11% polyacrylamide in the presence of 0.1% SDS. Extracellular virus was also labeled with L[^{35}S] methionine and compared to a control as described in the Results. Coomassie blue-stained virus polypeptides are as follows: (B) AcMNPV purified from the polyhedra of infected TN-368-10 cells, (C) and (D) extracellular virus purified from ^{35}S-cell protein + virus (control) or ^{35}S-virus + cell protein preparations, respectively. The autoradiogram of L[^{35}S]methionine-labeled polypeptides in slots C and D is shown in slots E and F, respectively. The molecular weights of the standard proteins, electrophoresed in slot A, are: phosphorylase a, 90,000; bovine serum albumin, 68,000; L-glutamic dehydrogenase, 53,000; DNA nuclease, 31,000; chymotrypsinogen, 25,000; and myoglobin, 17,200. The molecular weight times 10^{-3} of extracellular virus polypeptides are indicated to the right of the autoradiogram.

C. Competition RIA

Because of the complex structure of baculoviruses and problems associated with obtaining antigens of sufficient purity and quantity (Harrap et al., 1977; Summers and Smith, 1978; Bell and Orlob, 1978), immunological studies on purified individual structural polypeptides of the virion have not been reported. Most detailed studies have been confined to the major paracrystalline protein of the inclusion described as granulin for granulosis viruses and polyhedrin for nuclear polyhedrosis viruses. A single polypeptide of 25,000 to 31,000 molecular weight, polyhedrins and granulins have been shown by two-dimensional peptide mapping to possess both common and unique tryptic peptides (Summers and Smith, 1975, 1976; Maruniak and Summers, 1978). The presence of similar antigenic sites in these group related proteins have been documented by immunological analysis of several polyhedrins and granulins (Crozier and Meynadier, 1972a, b, 1973; Norton and DiCapus, 1976; Rohrman et al., 1978).

This portion of the study describes the efficacy of preparative polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) for isolating baculovirus structural polypeptides for use in immunological studies. Radioimmunoassay (RIA) is employed to compare the immunoreactivity of SDS purified AcMNPV polyhedrin with the reactivity of polyhedrin translated in infected cells showing that the detergent purified protein retains its immunological integrity. Using competitive inhibition studies as we have also investigated the immunoreactivity of highly purified granulins and polyhedrins from six baculoviruses to evaluate the ability of polyhedrin and granulin antisera to discriminate antigenic differences.

1. Immunodiffusion of polyhedrins and granulins

Preliminary immunological assessment of preparative SDS-PAGE purified polyhedrins and granulins using immunodiffusion assays resulted in the precipitin patterns presented in Figures 9 A-F. TnGV granulin reacted with homologous antiserum to form two major and one minor precipitin bands (Figure 9). SfGV granulin, with primary structure similar to that of TnGV (Summers and Smith, 1976) showed a reaction of identity with one major precipitin band. TnGV granulin antiserum also recognized related antigenic determinants in AcMNPV, RoMNPV, AgMNPV, and TnSNPV polyhedrins (Figures 9B and C). Under the experimental conditions utilized HaMNPV and HzSNPV polyhedrins did not appear to react with TnGV granulin antiserum (Figures 9B and C). In a similar experiment AcMNPV polyhedrin antiserum indicated common antigenic determinants between AcMNPV, RoMNPV, and TnSNPV polyhedrin. However, AcMNPV polyhedrin antiserum did not react with HaMNPV and HzSNPV polyhedrins or TnGV and SfGV granulins.

Because several laboratories have reported that protease activity does not alter the immunoreactivity of polyhedrins and granulins from several species of baculovirus (Kalmakoff et al., 1978; Norton and DiCapua, 1976), polyhedrins and granulins degraded by enzyme activity were also reacted with the TnGV granulin and AcMNPV polyhedrin antisera. The

immunodiffusion patterns for these proteins and TnGV granulin antiserum are shown in Figures 9D-F. While immunoreactivity of TnGV granulin with homologous antiserum is maintained, the reactions are diminished and patterns with other polyhedrin is noticeably reduced (Figure 9D), TnSNPV, and AgMNPV polyhedrin reactions are lost (Figure 9F). In contrast, HaMNPV and HzSNPV polyhedrins have similar antigenic determinants revealed by proteolytic hydrolysis (Figure 9E-F). Since alternations also occurred with AcMNPV polyhedrin serum tested against degraded polyhedrins and granulins (Data not shown), only undegraded SDS-PAGE purified antigens were used for further immunological testing with RIA.

2. RIA of AcMNPV polyhedrin and TnGV granulin

Titrations of AcMNPV polyhedrin and TnGV granulin antisera with homologous iodinated antigen showed that dilutions of 1:2600 (AcMNPV) and 1:6500 (TnGV) bound 50% of the respective labelled proteins. Serial dilutions of unlabelled AcMNPV polyhedrin and TnGV granulin were then employed to competitively inhibit binding of homologous labelled antigen at these antiserum dilutions to define assay parameters. (Figures 10A and B). The standard curve for AcMNPV polyhedrin showed that 80 pg of AcMNPV polyhedrin would reproductively inhibit binding by 10%, establishing the lower limit or sensitivity of the assay. Five hundred pg produced 50% inhibition and 3.0 ng were required for 90% inhibition (Figure 10A). The working range of the TnGV assay was 600 pg to 600 ng (Figure 10B).

3. RIA of AcMNPV polyhedrin synthesized in vivo

A quantitative assay of AcMNPV polyhedrin synthesized in vivo was made in infected TN-368 cells. The results (Figure 11) show that a competition curve prepared by use of 10-fold decreasing amounts of infected TN-368 cell lysates paralleled a standard curve obtained by adding SDS-purified AcMNPV polyhedrin to uninfected cell lysates. For each curve the essentially identical slopes produced in the linear regions of the assay show that SDS purified polyhedrin and polyhedrin synthesized in infected cells are recognized with equal affinity.

4. Competitive inhibition studies of polyhedrins and granulins

Since studies with AcMNPV polyhedrin in vivo indicated no loss in immunoreactivity of SDS-PAGE purified polyhedrin, a comparison was made of four polyhedrins and two granulins using competitive inhibition of ^{125}I-AcMNPV polyhedrin binding to AcMNPV polyhedrin antiserum. The results (Figures 12A and B) show that all polyhedrins and granulins assayed contain similar antigenic determinants for AcMNPV polyhedrin recognized by homologous antiserum. However, the quantity of such determinants varied among the proteins studied. While 0.5 ng of AcMNPV polyhedrin inhibited the binding of ^{125}I-AcMNPV polyhedrin by 50%, 5.0, 8.0, and 200 ng of RoMNPV, TnSNPV, and HaMNPV polyhedrins were required to achieve equivalent inhibition (Figure 12A). Fifty percent inhibition with HzSNPV polyhedrin and TnGV and SfGV granulins required 50, 55, and 220 ng

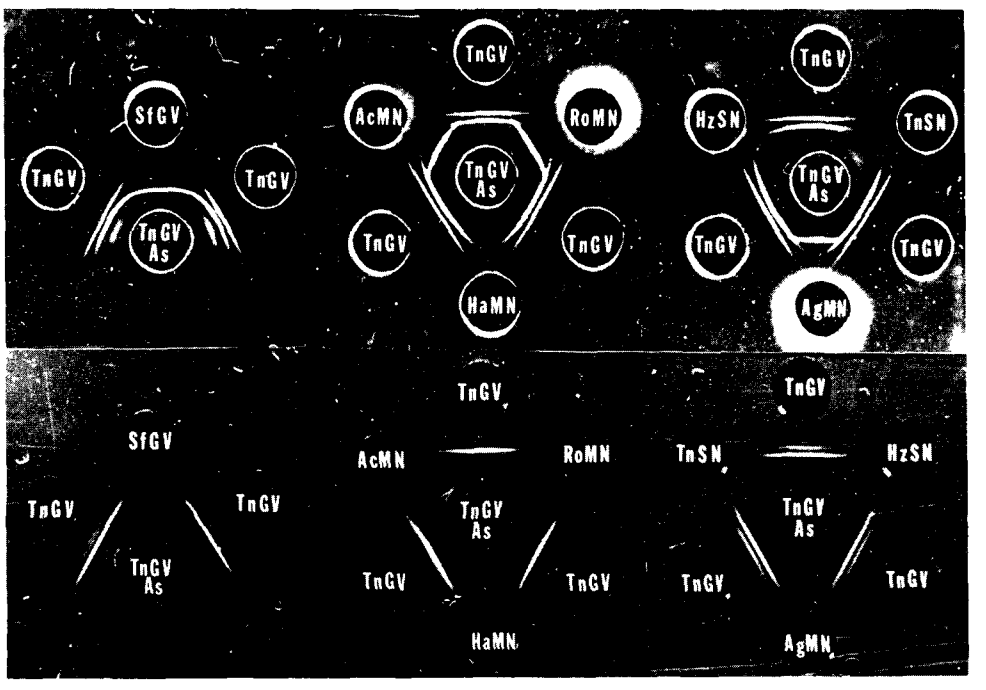

Figure 9. Immunodiffusion of polyhedrins and granulins. Trichoplusia ni granulin antiserum (TnGV As) was reacted with equivalent concentrations (15 µg/well) of undegraded (protease inactive, A-C) and degraded (protease active, D-F) polyhedrins and granulins. Plates were incubated for 24 h at room temperature and photographed directly. The polyhedrins and granulins used were TnGV, Trichoplusia ni granulin; SfGV, Spodoptera frugiperda, granulin; AcMN, Autographa californica polyhedrin; RoMN, Rachiplusia ou polyhedrin; HaMN, Heliothis armigera polyhedrin; HzSN, Heliothis zea polyhedrin; TnSN, Trichoplusia ni polyhedrin; AgMN, Anticarsa gemmitalis polyhedrin.

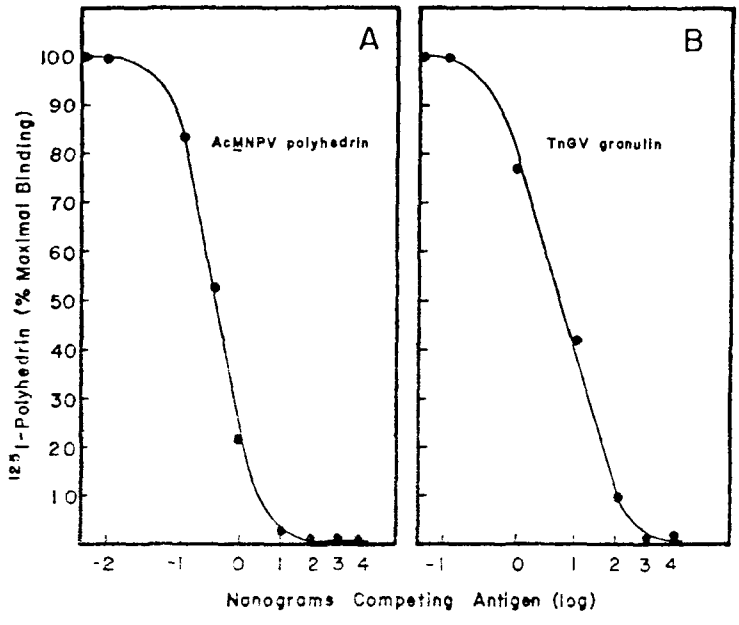

Figure 10. Radioimmunoassay of AcMNPV polyhedrin and TnGV granulin. Ten-fold dilutions of AcMNPV polyhedrin (A) and TnGV granulin (B) in quantities from 10 µg to 10 pg were reacted for 24 h with that dilution of homologous antiserum which bound 50% of 10,000 CPM of homologous iodinated antigen. 10,000 CPM of ^{125}I-AcMNPV polyhedrin of ^{125}I-TnGV granulin were added to each well, incubated an additional 24 h, the wells washed, and the % maximal binding determined as described in Materials and Methods.

respectively (Figure 12B). Also, the slopes of the inhibition curves for heterologous polyhedrins and granulins indicated that AcMNPV polyhedrin antiserum does not bind these proteins with comparable affinity (Figure 12A and B).

A similar experiment carried out with ^{125}I-TnGV and TnGV granulin antiserum resulted in the curves shown in Figure 13A and 13B, Seven nonograms of the homologous antigen were required to competitively inhibit by 50% the binding of ^{125}I-TnGV granulin to granulin antiserum. For comparison, 9.5 ng of SfGV granulin, 40 ng of AcMNPV polyhedrin, and 400 ng of TnSNPV polyhedrin were necessary to produce 50% inhibition (Figure 13A). RoMNPV, HzSNPV, and HaMNPV polyhedrins required 45 ng, 330 ng, and 4 µg for the same level of inhibition (Figure 13B). The possibility of greater degrees of inhibition for TnSNPV, HzSNPV, and HaMNPV polyhedrins was not tested since the upper limit of the assay was 10µ. Of particular note is the failure of SfGV granulin to effect complete inhibition of ^{125}I-TNGV granulin at high antigen concentrations (Figure 13A).

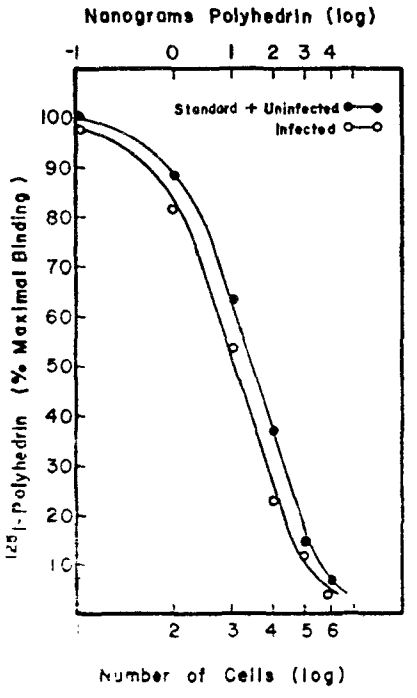

Figure 11. Detection of AcMNPV polyhedrin in infected cells. Ten-fold dilutions of TN-368 infected cell lysates (o-o) from 10^6 cells were prepared as described in Materials and Methods and reacted with AcMNPV polyhedrin antiserum at the dilution which bound 50% of the iodinated AcMNPV polyhedrin indicator. Mock infected cell lysates (•-•) from 10^6 cells diluted in a similar manner containing 10 µg to 10 pg of highly purified AcMNPV polyhedrin served to construct the standard curve. Following 24 h incubation, 10,000 CPM ^{125}I-AcMNPV polyhedrin were added, the incubation continued for 24 h, and % maximal binding determined. A parallel study of the detection of AcMNPV polyhedrin with respect to time indicated that 1 ng of polyhedrin could be detected at 12 h post infection in 10^3 cells, and a ten-fold increase in cell lysate (10^4 cells) resulted in a ten-fold increase in antigen concentration.

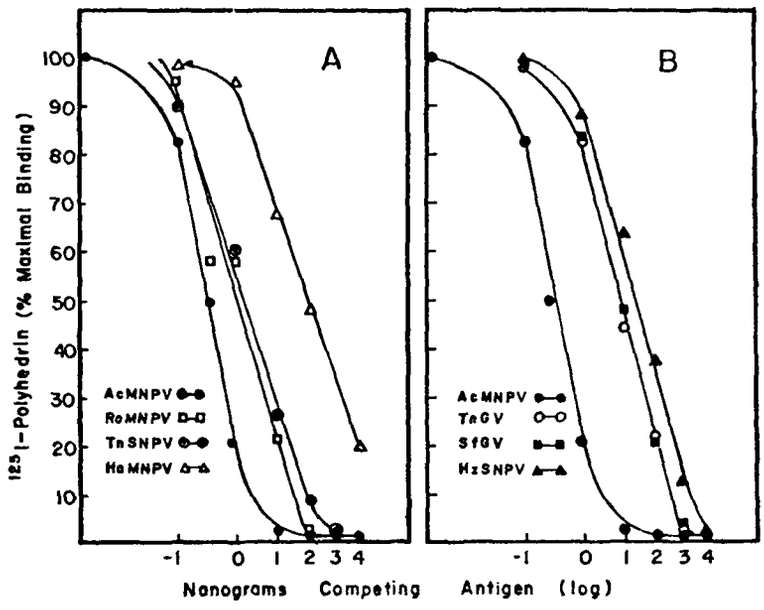

Figure 12. Comparison of polyhedrins and granulins by competitive RIA. Ten-fold dilutions of purified polyhedrins and granulins ranging from 10 µg to 10 pg were reacted with the dilution of AcMNPV polyhedrin antiserum binding 50% of iodinated homologous antigen. After incubation for 24 h, 10,000 CPM of ^{125}I-AcMNPV polyhedrin was added, and the ability of various polyhedrins and granulins to inhibit binding was determined and expressed as % maximal binding in the absence of competing antigen. The competing antigens used were: AcMNPV polyhedrin (●-●), RoMNPV polyhedrin (▫-▫); TnSNPV polyhedrin (◎-◎); HaMNPV polyhedrin (△-△); TnGV granulin (○-○); SfGV granulin (■-■); and HzSNPV polyhedrin (▲-▲).

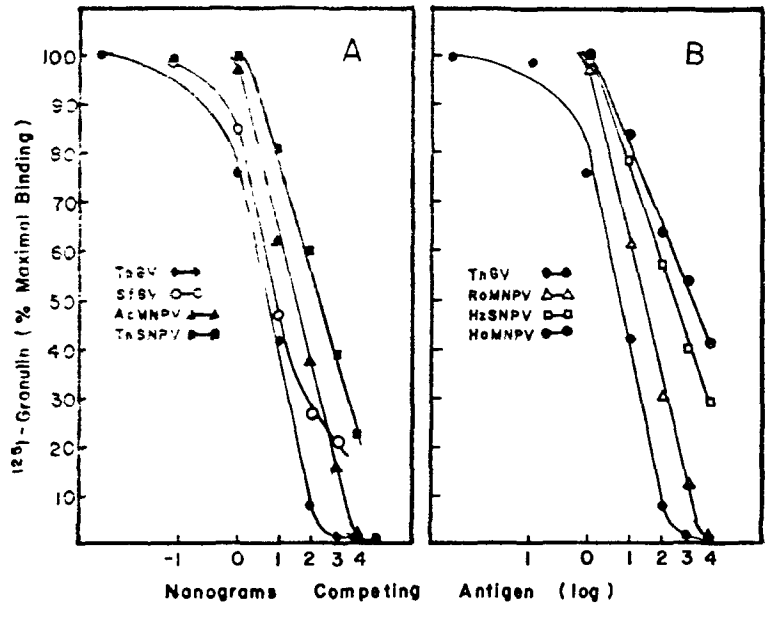

Figure 13. Comparison of polyhedrins and granulins by competitive RIA utilizing TnGV granulin antiserum. Ten-fold dilutions of purified polyhedrins and granulins ranging from 10 µg to 10 pg were tested for the ability to inhibit the binding of 10,000 CPM of ^{125}I-TnGV granulin to homologous antiserum. The results are expressed as % maximal binding in the absence of competing antigen. The polyhedrins and granulins used were: TnGV granulin (●-●); SfGV granulin (○-○); AcMNPV polyhedrin (▲-▲); TnSNPV polyhedrin (■-■); Ro MNPV polyhedrin (△-△); HzSNPV polyhedrin (□-□); and HaMNPV polyhedrin (◎-◎).

5. Discussion

This study documents the successful utilization of preparative SDS polyacrylamide gel electrophoresis to isolate and purify sufficient quantities of a major baculovirus structural protein for immunological studies. Further, the identical slopes observed in standard (SDS-PAGE purified) and unknown (cell lysate) competition curves indicates that no loss of affinity has occurred as a result of SDS-PAGE purification (Figure 11). Preparative SDS-PAGE also circumvents several basic problems associated with the serology of baculoviruses and baculovirus proteins. Antigen purity (Bell and Orlob, 1977) and potential effects of degradation by an inclusion associated protease (Eppstein and Thoma, 1975; Kozlov et al., 1975; Summers and Smith, 1975, 1976) represent two areas of difficulty, particularly for the study of polyhedrins and granulins. Altered immunoreactivity of protease degraded polyhedrins or granulins was evidenced by altered immunodiffusion reactions (Figure 9D-F) and by reduced sensitivity and affinity in RIA (Data not shown).

Two-dimensional high voltage electrophoresis of polyhedrins and granulins have shown all proteins analyzed contained varying numbers of similar tryptic peptides (Summers and Smith, 1976; Summers and Maruniak, 1978). Each polyhedrin or granulin, however, also contained peptides unique in migration. TnGV and SfGV granulins were very similar, which is confirmed in both immunodiffusion (Figure 9A) and competitive inhibition studies (Figure 13A). Less similar polyhedrins by tryptic peptide analysis also demonstrate a large spur formation in immunodiffusion with TnGV granulin (Figure 9B-C). This result indicates that common antigenic determinants exist on all polyhedrins and granulins, but that some proteins, in this case TnGV granulin, have other antigenic determinants which are also available to the immune system. No such distinction can be seen with AcMNPV polyhedrin antiserum.

Our results from competitive inhibition studies using RIA further characterized the "group antigen" of polyhedrins and granulins. Polyhedrins and granulins were shown to be heterologous with respect to the numbers of antigenic determinants per molecule of protein. For example, HaMNPV and HzSNPV polyhedrin did not react with TnGV granulin antiserum in immunodiffusion (Figure 9B-C). When 500 and 1000 fold greater concentrations of these proteins were used as competitors in RIA, inhibition of binding of homologous serum occurred. Thus, the more sensitive immunological assay could measure very small numbers of determinants in HaMNPV and HzSNPV polyhedrins by enzyme hydrolysis which are apparently not accessible to antibody in the undegraded molecule (Figure 9E and 9F). Also, aggregation of polyhedrin or granulin to higher molecular weight complexes may eliminate antigenic determinants formerly accessible to antibody.

In addition to quantitative differences in polyhedrin and granulin "group antigens", qualitative differences in the structure of antigenic determinants were shown to exist. This is suggested by the reduction in slopes of heterologous competition curves, indicating less affinity for antibody binding sites compared to the homologous antigen (Figure 12 and 13).

Because of the serological similarities identified among apparently closely and distantly related baculoviruses shown in this study, it will be difficult to use the present application for identification of baculovirus isolates, strains, etc. However, the use of preparative SDS polyacrylamide gel electrophoresis for the purification of other baculovirus structural polypeptides in conjunction with immunological analysis by competition RIA should provide means to develop sensitive and specific serological probes for the study of the serology of baculovirus structural proteins relative to their identity and role in biological specificity.

6. Micro-SPIRA: Enveloped Nucleocapsids

The relative binding of ^{125}I-labelled AcMNPV LOVAL IgG increased significantly with a concomitant increase in antigen concentration (Fig. 16) within the range of 1-200 ng. At this level there was little if any significant competition of PMB-NOV and polyhedrin as compared to homologous binding.

AcMNPV EV was titrated with homologous antiserum and [^{125}I]IgG (Figure 17). The apparent sensitivity of the homologous binding was approximately 10 ng. The assay appeared to be specific for AcMNPV EV at concentrations of 10-200 ng of antigen. The results of the EV binding assay exhibit similar sensitivity and specificity as did the LOVAL assay.

The binding curve for AcMNPV polyhedrin showed that the sensitivity of this assay allowed the detection of as little as 200 pg of homologous antigen. The results of this assay show that the antiserum is specific for the homologous antigen to at least 200 ng (Figure 18).

Using LOVAL antisera, the ability of unlabelled antisera to compete with [^{125}I]IgG is shown in Figure 19. Comparisons were made with purified LOVAL IgG, polyhedrin, and EV antisera. The results of this qualitative assay indicate the presence of common antigenic determinants among EV, polyhedrin, and LOVAL.

In the present preliminary study, a micro-SPIRA was successfully developed at a sensitive level for the detection of AcMNPV enveloped nucleocapsids, alkali-liberated virus (LOVAL), extracellular virus EV, and highly purified polyhedrin. The sensitivity of the micro-SPIRA was 5-10 ng for AcMNPV LOVAL and EV. By comparison, the polyhedrin assay was 10-100 times more sensitive. These assays were specific for the detection of homologous antigens to the level of 200 ng.

Micro-SPIRA was used to demonstrate the ability of AcMNPV LOVAL, purified IgG, EV, and polyhedrin antisera to inhibit the specific binding of AcMNPV LOVAL [^{125}I]IgG. In a comparison of the degree of inhibition of the homologous antiserum and IgG, the results likely reflect differences in antibody titers as a result of purification. The inhibition of EV and polyhedrin antisera may also involve differences in antibody titers of specific antibody to determinants in common with AcMNPV LOVAL. However, the data may also indicate that this inhibition reflects differences between specific antibodies against related antigenic determinant(s)

on AcMNPV LOVAL. Therefore, the results of this study show the ability to detect specific antiviral antibodies from different sources.

D. Biological Properties of Infectious Virions

One of the significant observations made in early studies is that virus, as isolated from purified polyhedra, is relatively noninfectious in cell culture, whereas extracellular, nonoccluded virus recovered from the hemolymph of infected insects or medium from infected cell cultures is highly infectious. In one study (Summers and Volkman, 1976), we have clarified the nature of the nonoccluded infectious virus by making biophysical and morphological comparisons of the nonoccluded infectious material from insect hemolymph and cell culture medium.

Electron microscope examination and buoyant density profiles of nonoccluded Rachiplusia ou and Autographa californica nuclear polyhedrosis viruses purified from both infectious insect hemolymph and cell culture medium revealed that the viruses are enveloped, single nucleocapsids. The envelopes exhibited variation in the amount and the degree of fit with regard to the nucleocapsids. This was determined by: (i) electron microscope observations of virus budding from the surface of infected cells; (ii) electron microscopic observations of negatively stained preparations of pelleted, highly purified, nonoccluded enveloped particles; and (iii) the resolution and density distributions of nonoccluded virus in sucrose gradients after centrifugation to equilibrium; all were compared with virus extracted from polyhedra. Peplomers, observed on the surface of enveloped nucleocapsids of nonoccluded virus, are not associated with polyhedra-derived virus. Density gradient analysis indicated that virus from insect hemolymph and culture medium exhibited similar densities of approximately 1.17 to 1.18 g/ml. This is significantly different from the bouyant density of an alkali-liberated, enveloped single nucleocapsid (1.20 g/ml). Results of this study show that the nonoccluded forms of two nuclear polyhedrosis viruses from two different sources, hemolymph and cell culture, are similar with regard to several morphological and biophysical characteristics but are quite different from the alkali-liberated polyhedra-derived form of the virus.

Physical-infectious particle ratio. The physical-infectious particle ratios for AcMNPV LOVAL and EV are given in Table V. It was determined that AcMNPV LOVAL had a physical-infectious particle ratio of about 2.2 x 10^5:1 for the bundles and 2.4 x 10^5:1 for the enveloped single nucleocapsids, as assayed by the plaque assay in vitro. In contrast to this, the AcMNPV EV had a physical-infectious particle ratio of 128:1. If one can assume that the DNA-protein ratio is the same for the various forms of RoMNPV as it is for the analogous forms of AcMNPV, then the calculated physical-infectious particle ratios for these forms are very similar as well (1.6 x 10^5:1 for bundles; 2.9 x 10^5:1 for singles). To test whether the necessary exposure to alkaline conditions of the LOVAL preparations could account for their relatively greatly diminished infectivity, a sample of cell culture-derived extracellular nonoccluded AcMNPV was taken through the alkaline solubilization procedure and then tested for infectivity. The physical-infectious particle ratio was increased about two-fold (to 256:1).

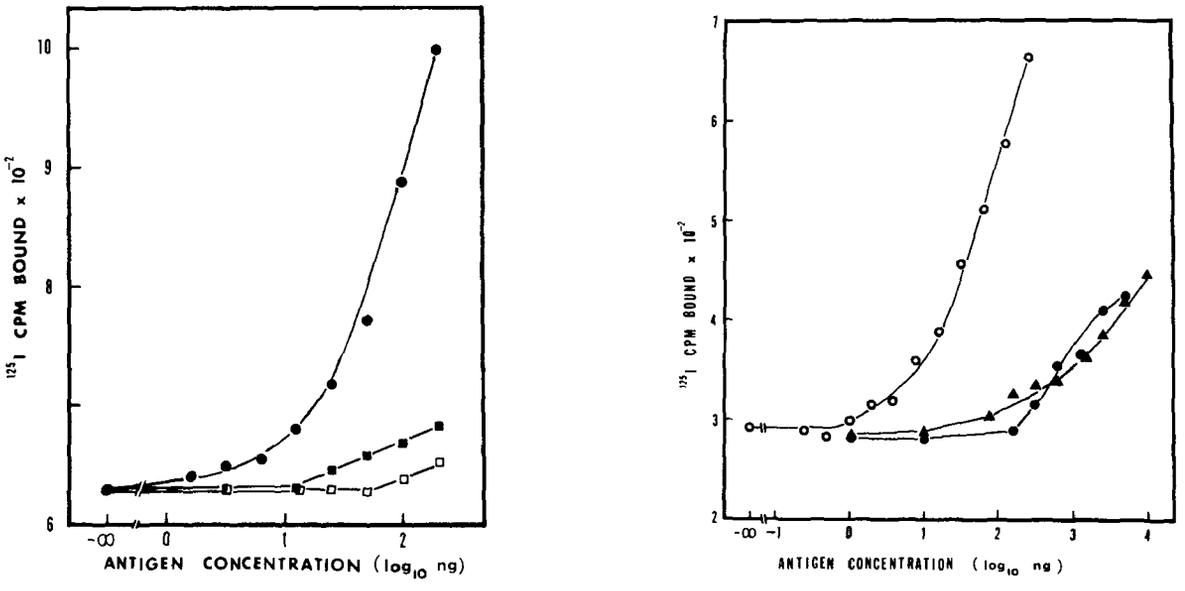

Figure 16. Micro-SPIRA for enveloped nucleocapsids. AcMNPV LOVAL (●), EV (■), and polyhedrin (□), were titrated by using AcMNPV LOVAL antiserum and AcMNPV LOVAL [^{125}I]IgG (41 ng with a specific activity of 3 μCi/μg). Microtiter wells were coated with a 1:10 serum dilution at 4°C for 4 hr and then were coated with 2% BSA solution at 4°C overnight. A serial dilution of each antigen was added to the wells and was allowed to set at 4°C overnight. [^{125}I]IgG was added to each well. The microtiter plates were allowed to set at 37°C for 4 hr, rinsed and counted.

Figure 17. Micro-SPIRA for AcMNPV PMB-NOV. Enveloped nucleocapsid preparations and polyhedrin were tested in the assay system using AcMNPV EV antiserum and [^{125}I]IgG (29 ng with a specific activity of 4μCi/μg). Microtiter wells were coated with a 1:10 serum dilution using the conditions described in Fig. 1. The antigens used were AcMNPV EV (O), AcMNPV LOVAL (●), and AcMNPV polyhedrin (▲).

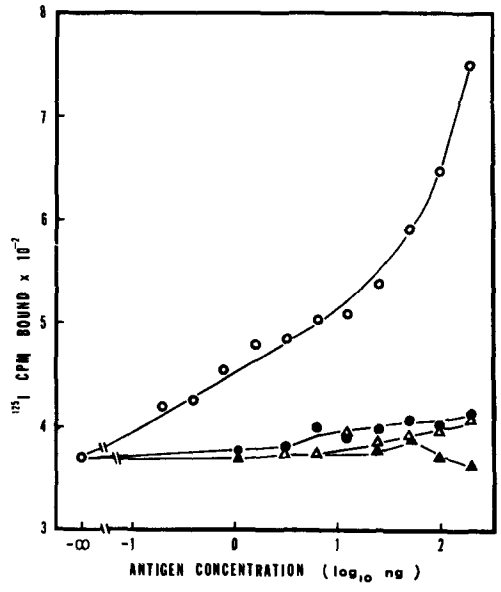

Figure 18. Micro-SPIRA for AcMNPV polyhedrin. AcMNPV polyhedrin and enveloped nucleocapsids were compared using AcMNPV polyhedrin antiserum (1:10 dilution) and [^{125}I]IgG (26 ng with a specific activity of 4μCi/μg). The assay procedure was the same as previously described in Fig. 1. Antigens tested were: AcMNPV polyhedrin (O), AcMNPV LOVAL (●), AcMNPV EV (▲). Preimmune serum (Δ) was used as a control and demonstrated no binding.

Figure 19. Micro-SPIRA for the detection of specific antiviral antibodies. Microtiter wells were coated with a 1:10 dilution of AcMNPV LOVAL antiserum at 4°C for 4 hr and then were coated with the 2% BSA solution at 4°C overnight. One-hundred ng of AcMNPV LOVAL was added to each well and was incubated at 4°C overnight. Fivefold dilutions of each antiserum and IgG were added to the wells. After incubation at 37°C for 4 hr, the wells were washed with buffer, and AcMNPV LOVAL [^{125}I]IgG (33 ng with a specific activity of 3 μCi/μg) was added and incubated at 37°C for 4 hr. AcMNPV LOVAL (O); AcMNPV LOVAL IgG (●). IgG concentration in the undiluted solution was 1.0 mg/ml; AcMNPV EV (□); AcMNPV polyhedrin (■); and preimmune serum (Δ).

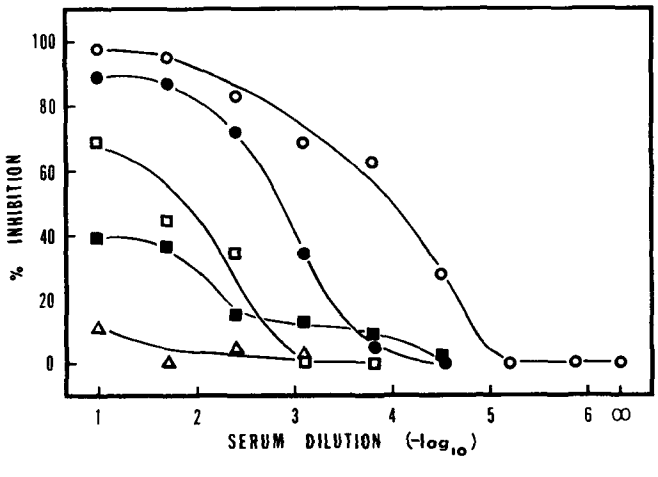

Table V. AcMNPV physical-infectious particle ratios as assayed in vitro (Summers and Volkman, 1976)

Virus prepn	DNA		Particle/ μg of protein ($\times 10^9$)	PFU/μg of protein	Particles/ PFU
	μg/μg of protein	g/μg of protein ($\times 10^{-7}$)			
PMB-NOV	0.19	1.9	1.1	8.9×10^6	1.28×10^2
LOVAL I[a]	0.20	2.0	1.2	5.0×10^3	2.40×10^5
LOVAL II-X[b]	0.30	3.0	1.8	8.4×10^3	$2.20 \quad 10^5$

[a]Single nucleocapsid per envelope
[b]Many nucleocapsids per envelope

To further characterize the biological differences between NOV and LOVAL we conducted a study wherein we compared quantitatively the infectivity of AcMNPV LOVAL and EV as assayed both in vitro and in vivo, the latter as administered by both oral and intercoelomic injection (Volkman and Summers, 1977).

Table VI gives the LD_{50} and the $TCID_{50}$ values for the various preparations under the different conditions. The results show that by the per os route of assay, EV is very low in infectivity: 1×10^5-fold less infectious than it is by in vitro assay. When injected into the hemocoel, however, it is much more highly infectious and approaches the level obtained in the in vitro assay. A further extension of this logic predicts that LOVAL should be more highly infectious by per os administration in vivo than by coelomic administration or by in vitro assay. The results in Table VI, however, do not support this conclusion. They show that

Table VI. LD_{50} and $TCID_{50}$ values of LOVAL and EV

Virus prepn	Route of admins	Per LD_{50}		Per $TCID_{50}$		Particles/ LD_{50} + Particles/ $TCID_{50}$
		Purified viral protein (μg)	Number of particles (genome equiv)[a]	Purified viral protein (μg)	Number of particles (genome equiv)[a]	
LOVAL	Per os	1.28×10^{-5}	2.30×10^4	1.16×10^{-4}	2.09×10^5	1:9
LOVAL	Hemocoelic	3.92×10^{-5}	7.05×10^4	1.16×10^{-4}	2.09×10^5	1:3
PMB-NOV	Per os	8.00×10^{-3}	8.80×10^6	7.84×10^{-8}	8.60×10^1	1×10^5:1
PMB-NOV	Hemocoelic	2.49×10^{-7}	2.74×10^2	7.84×10^{-8}	8.60×10^1	3:1

LOVAL is only nine-fold more infectious by the per os route than by assay in vitro and that the per os route is only three-fold more efficient than the hemocoelic route. These differences are not comparable to the differences observed with EV.

In order to investigate whether LOVAL's consistent low efficiency of infection, even when administered per os, was due to artificial removal of the polyhedrin protein, we assayed highly purified whole polyhedra by per os infection and found the LD_{50} to be about 120. If the efficiency of infection was the same for the occluded virions as it was for LOVAL, there would be about 190 genome equivalents per polyhedrin. The results indicate, then, that occluded virions are more infectious than LOVAL when administered per os, though only slightly so, about five-fold.

It appears, therefore, that the virions that become occluded, when assayed either as LOVAL or as occluded virus, are innately less infectious than EV. There is a 50-fold difference when comparing infectivity under the best conditions of each.

From this, the results suggest that, whereas the nonoccluded form of the MNPVs derived from hemolymph and CCM are similar, they are very different from the alkaline-liberated form of the occluded MNPVs. One might expect serological differences among viruses from the three sources, and indeed there are. We have found (Volkman et al., 1976) that antisera raised against alkali-liberated virus inhibits in vitro plaque formation by homologous virus, but does not interfere with plaque formation by the nonoccluded form. These data suggest the necessity of biochemical, biophysical, and related serological comparisons of virus not only from different sources in the same host, but also from different sources in alternate hosts, to detect any virus modifications and, once detected, to determine the nature of virus structure relative to these modifications.

E. Immunoperoxidase

1. Production of enveloped nucleocapsids, polyhedrin and polyhedra

Time course studies were undertaken wherein the onset and the rate of increase in infected cells of each of the following, as detected by immunoperoxidase reaction, were compared: synthesis of enveloped nucleocapsid antigens, production of infectious intracellular and extracellular nonoccluded virus, synthesis of polyhedrin, and the formation of polyhedra. Enveloped nucleocapsid antigens were first detected in infected TN-368-10 cells at 8 h post infection (Figure 14), but not at 6 h. The first evidence of infectious progeny virus (both intra- and extracellular) as detected by polyhedral plaque assay, however, was at 10 h post infection (p.i.): a lag of about 2 h between detection of enveloped nucleocapsid antigens and that of infectious virus.

Polyhedrin was first detected at 12 h p.i. This shows that polyhedrin is synthesized late relative to enveloped nucleocapsid proteins, and its presence did not precede that of infectious virus (Figure 15a). However, the total amount of infectious nonoccluded virus, both intra- and extracellular, began to decline at the onset of polyhedrin production between 12 and 14 h p.i. (Figure 1 and 2).

The crystallization of polyhedrin into polyhedra was initially observed to occur at 15 h p.i. The rate of production of polyhedra in infected TN-368-10 cells paralleled the rate of polyhedrin synthesis as judged by immunoperoxidase reaction with an approximate lag time of 1 to 3 h (Figure 15a and b). In infected TN-368-13 cells, however, the rate of production of polyhedra was less than that of polyhedrin (Figure 15b). This difference in the rate of appearance of polyhedra between infected TN-368-10 cells and TN-368-13 cells has been noted before (Volkman and Summers, 1976) and presumably is related to the difference in their abilities to serve as indicator cells in the plaque assay (Volkman and Summers, 1975) and to the difference in their EV yields (Volkman *et al.*, 1976).

2. Intracellular location of enveloped nucleocapsid and polyhedrin antigens

With the LOVAL antiserum the first immunoperoxidase staining in infected cells was observed at 8 h p.i. in distinct regions of the cytoplasm indicating the site of enveloped nucleocapsid antigen synthesis and accumulation. The staining was most intense near the nucleus, but no stain was detected in the nucleus at that time (Summers *et al.*, 1978).

At 10 h after infection of TN-368-10 cells, LOVAL antiserum produced, in addition to the cytoplasmic staining present at 8 h, a very slight reaction uniformly distributed throughout the nucleus indicating the presence of some enveloped nucleocapsid antigen(s). This coincided with the detection of both intracellular and extracellular infectious virus.

At 12 h p.i., approximately 75% of the TN-368-10 cells treated with LOVAL antiserum were uniformly stained, although the staining was slightly

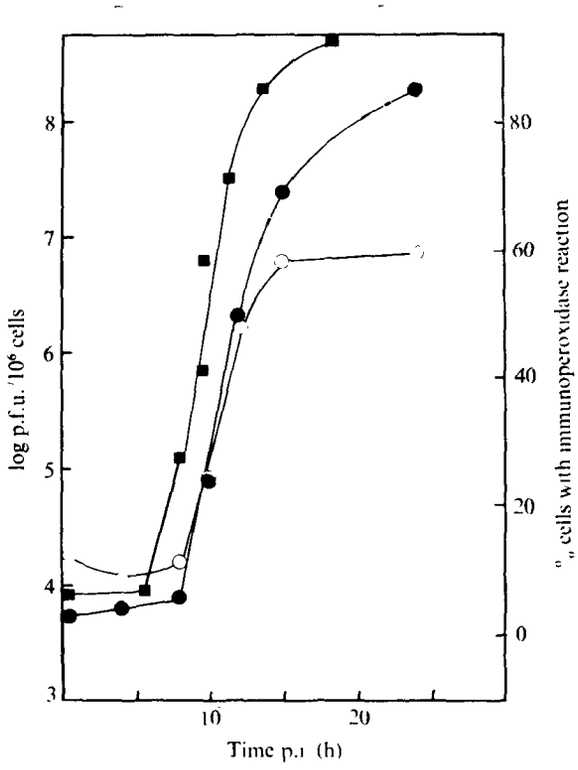

Figure 14. Accumulation of intra- and extracellular infectious virus of enveloped nucleocapsid antigens detected by the plaque assay and immunoperoxidase technique. ■-■, Percentage of cells in which enveloped nucleocapsid antigens were detected. Virus titres: ●-●, extracellular; o-o, intracellular virus. Infectivity was determined by back titration of cell culture medium or disrupted cells in TN-368-10 cells.

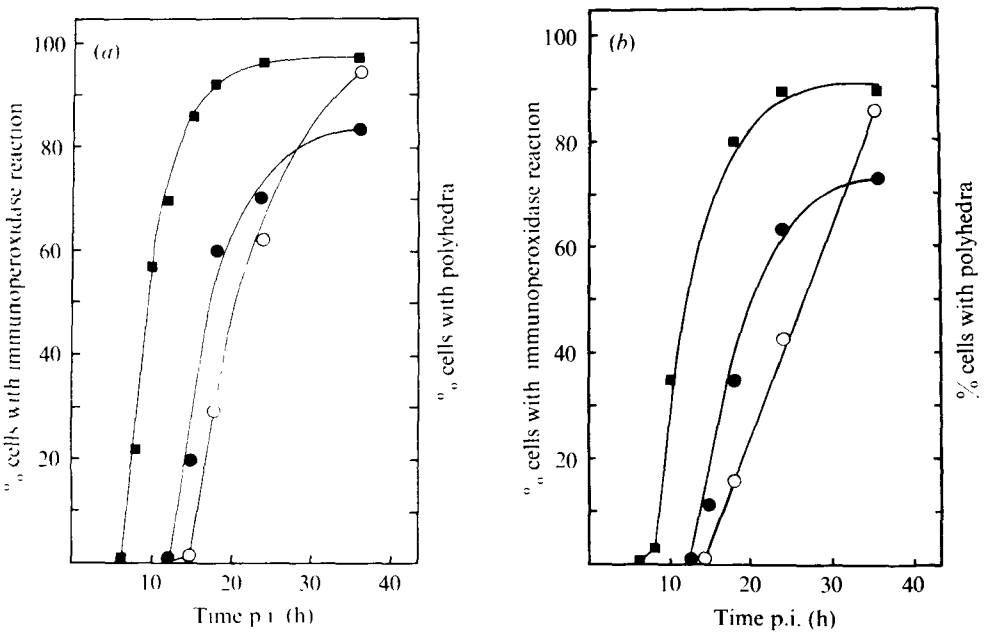

Figure 15. Production of TN-368-10 (a) and TN-368-13 (b) cells of AcMNPV enveloped nucleocapsid and polyhedrin antigens, as detected by the immunoperoxidase technique, and of polyhedra. The percentage of cells was determined that reacted with AcMNPV LOVAL (■) or AcMNPV polyhedrin (●) antisera, or contained polyhedra (○).

more intense in the nucleus than the cytoplasm. At this time, less than 10% of the infected cells became stained with polyhedrin antiserum. The intensity of staining in the cytoplasm of infected TN-368-10 cells using LOVAL antiserum began to decrease when the cells first stained with polyhedrin, i.e., about 12 h p.i.

At 15 h p.i., 86% of the cells reacted with the LOVAL antiserum, giving intense staining in the nucleus particularly in the virogenic stroma. Markedly less staining was observed in the cytoplasm indicating a possible shutdown in the synthesis of enveloped nucleocapsid antigens. At 15 h the distribution of polyhedrin was similar to that at 12 h, but the staining was slightly more intense. Twenty per cent of the cells reacted with the polyhedrin antiserum, but intact polyhedra were observed in only 25% (Summers et al., 1978).

At 18 h p.i., 92% of the infected TN-368-10 cells reacted with LOVAL antiserum giving a somwhat even distribution of stain throughout the nucleus, with the exception of some localization of stain adjacent to the inner nuclear membrane. There was greatly diminished staining in the cytoplasm. When treated with polyhedrin antiserum, 60% of the cells distinctly stained in the nucleus while reaction in the cytoplasm was significantly diminished. The staining appeared to be most intense near the inner nuclear envelope. Polyhedra were observed in 30% of the cells.

At 24 h p.i., staining in TN-368-10 cells treated with LOVAL antiserum was similar to that at 18 h except that cells containing numerous polyhedra in the nuclei reacted less intensely. When polyhedrin antiserum was used, 70% of the cells stained intensely in the nucleus with some apparent localization of stain near the inner nuclear membrane; staining was markedly reduced in the cytoplasm. Polyhedrin were observed in 62% of the cells.

At 36 h to 42 h p.i., when over 95% of the cells contained polyhedra, TN-368-10 cells treated with LOVAL antiserum showed only slight staining in the cytoplasm and a marked reduction of stain in the nucleus. These results indicated that as enveloped nucleocapsids became occluded, the reaction with LOVAL antibodies decreased.

TABLE IV: Detection of AcMNPV antigens and infectivity by immunoperoxidase and plaque assay in insect cell lines

	Cell lines				
	Bombyx mori	Carpocapsa pomonella	Manducta sexta	Mamestra brassicae	TN-368-10
Time p.i. (h) ...	42	42	18	18	18
% cells Virus	90%	0	55%	71%	42%
indicating Polyhedrin	<1.0%	0	2%	29%	61%
antigens					
Infection obtained by back titration in TN-368-10 cells	Yes	No	Yes	Yes	--

3. Infection of Manduca sexta, Mamestra brassicae, Bombyx mori 5 and Carpocapsa pomonella cells

Comparisons of several different invertebrate cell lines were made in order to test the general utility of the immunoperoxidase technique as developed with TN-368-10 cells and TN-368-13 cells (Table IV). Although time course studies were not conducted, antisera to enveloped nucleocapsids and polyhedrin tested against M. brassicae cells at 18 h p.i. showed reactions typical of those observed in TN-368-10 and TN-368-13 cells. It is now known from other studies in this laboratory that the M. brassicae cell line yields titres of EV and polyhedra at least comparable to those produced by TN-368-10 cells. Carpocapsa pomonella cells are resistant to infection by AcMNPV and no progeny virus was detected by back titration of cell culture medium in TN-368-10 cells. Also, neither conjugated antiserum for enveloped nucleocapsids nor for polyhedra reacted with these cells at time intervals up to 42 h post inoculation.

In B. mori cells, different results were obtained. At 42 h p.i., 90% of the cells reacted with LOVAL antiserum. Staining was more intense in the cytoplasm adjacent to the outer nuclear membrane with some very slight staining in the nucleus. The cells did not react, however, with polyhedrin antiserum. Inspection of 400 cells revealed that only 0.5 to 1.0% contained polyhedra. By 42 h p.i., cells yielded titres of 10^6 p.f.u./ml or more of nonoccluded virus particles as confirmed by back titration of B. mori cell culture medium in TN-368-10 cells.

4. Discussion

The immunoperoxidase technique can be used to monitor infected invertebrate cells for the presence of virus antigens during the course of infection. Some of the advantages that this technique offers over the popular plaque assay and other polyhedrin-dependent methods are: (1) it is independent of the production of polyhedra and may be used to detect enveloped nucleocapsid associated antigens or alternatively, polyhedrin antigens directly, and (2) it can detect virus antigens in the absence of infectious virus and polyhedrin. Thus, the method can be used in the search for: (1) virus mutants which under certain conditions either cannot produce polyhedrin or produce an altered polyhedrin that cannot crystallize into polyhedra, or (2) virus mutants that do not produce infectious particles in certain cell types. If used in conjunction with polyhedral plaque assay, the method could further the understanding of the mechanisms involved in the replication and production of occluded and nonoccluded virus.

It is difficult to evaluate the sensitivity of the assay except, perhaps, by comparing the sensitivity of antisera when used in this method with that of the same sera when used in radioimmunoassay. The polyhedrin antiserum used in this study could detect 75 pg of polyhedrin by binding studies. Furthermore, both competition radioimmunoassay and the immunoperoxidase technique first indicated the appearance of polyhedrin at 12 h p.i. By radioimmunoassay, polyhedrin was detected by 12 h in as few as 100 cells; at the same time of infection, the immunoperoxidase technique

detected polyhedrin in 1 of 100 cells.

The LOVAL antiserum could detect 1 ng of virus, perhaps less by competition radioimmunoassay (M. D. Summers, unpublished results). If it were assumed that similar sensitivity existed for the immunoperoxidase method as for the radioimmunoassay, as compared to the polyhedrin studies, it could be estimated that 1 ng of enveloped nucleocapsid antigens could be detected, which represents, at a maximum, roughly 1.8 x 10^6 LOVAL particles (Volkman et al. 1976). This is probably an overestimation since the calculation is based upon total virus protein. Although not yet determined, the antiserum probably recognized only a small proportion of the structural proteins of the enveloped nucleocapsid.

The study relating time, presence and cellular location of antigen-antibody reaction revealed several things about the nature of AcMNPV infection of TN-368-10 and TN-368-13 cells. The fact that enveloped nucleocapsid antigens were detected 2 h in advance of infectious virus, both of which, in turn, were detected 2 to 4 h before polyhedrin, indicates that the synthesis of polyhedrin (in excess of our limits of detection) is not necessary for the production of infectious nonoccluded virus. The synthesis of infectious virus began to decline at the onset of polyhedrin production as detected by immunoperoxidase staining between 12 and 14 h p.i. This confirms our previous results (Volkman and Summers, 1975). Polyhedrin was synthesized about 2 h before the appearance of polyhedra. While the rate of appearance of polyhedra paralleled the rate of appearance of polyhedrin as evaluated by similar slopes (Figure 2) in TN-368-10 cells, the rate of appearance of polyhedra in TN-368-13 cells was less when compared to polyhedrin. As the rate of synthesis of polyhedrin was approximately the same for TN-368-10 and TN-368-13 cells (see slopes of reaction curves) (Volkman and Summers, 1975, 1976; Volkman et al., 1976), perhaps a factor involved in the crystallization of polyhedrin into polyhedra is related to the two cells lines' abilities to produce different quantities of polyhedra and at different times. The rate of appropriate enveloped nucleocapsid protein(s) to serve as a nucleation site.

Enveloped nucleocapsid antigens initially appeared in the cytoplasm of infected cells and were transported to the nucleus by 10 h p.i. At 15 h intense staining occurred in the nucleus while staining in the cytoplasm was greatly diminished. Intense staining and localization coincided with the appearance of the virogenic stroma (Volkman et al. 1976) just prior to polyhedrin formation. The intense staining associated with the virogenic stroma may be indicative of the insertion of LOVAL specific proteins into intranuclear virus envelopes. As the number of polyhedra per nucleus increased between 18 and 42 h, staining of enveloped nucleocapsid antigens in the nucleus decreased.

The testing of several invertebrate cell lines indicated that the immunoperoxidase technique could indeed be useful for host range studies. In addition, the peroxidase results with infected Bombyx mori 5 cells revealed that no intense intranuclear staining occurred using LOVAL or polyhedrin antisera, by contrast with TN-368-10 and TN-368-13 cells. In fact, the slight staining observed at 10 h p.i. in B. mori cells was sim-

ilar to that at 8 and 10 h p.i. in TN-368-10 cells. One can speculate that (1) certain LOVAL specific antigens responsible for the serological reaction are not synthesized in B. mori cells; (2) the transport of LOVAL specific antigens to the nucleus is inhibited; (3) LOVAL specific proteins are synthesized and transported to the nucleus, but fail to be inserted into the LOVAL envelope so that the antigenic structure recognized by the antibodies is not produced; (4) an interfering agent not detected by our technique may be present. Any of these possibilities may be directly or indirectly responsible for the lack of nuclear staining.

The observations raise the question of how the B. mori cell is able to block some function related to the synthesis of polyhedrin (Summers and Smith, 1976), but not replication of enveloped nucleocapsids. The infectious virus recovered from medium of infected B. mori 5 and B. mori I (from D. Knudson) infects TN-368-10 cells normally and therefore does not appear to be defective. In addition, extracellular virus from AcMNPV infected B. mori I cells can be 98.5% neutralized by homologous antiserum. Moreover, no plaque forming virus was detected when uninfected B. mori cells were sonicated and titrated for virus in TN-368-10 cells.

The finding that infected Bombyx mori cells produced enveloped nucleocapsid antigens and infectious virus in the absence of detectable polyhedrin and, consequently, polyhedrin production in nearly all the cells, illustrates precisely why polyhedrin dependent assays should only be used as indicators of virus infectivity in well characterized systems. There is therefore a need for more specific and sensitive techniques for the direct detection and quantification of enveloped nucleocapsids. The development of the immunoperoxidase technique as applied herein is one of the first steps in that direction.

F. Neutralization

Antisera raised against AcMNPV and RoMNPV LOVAL were tested for neutralization activity against AcMNPV and RoMNPV from various sources. The results of these experiments were that the antisera neutralized the homologous and heterologous LOVAL (Figure 20). It did not neutralize other forms of the virus, however, such as EV, alkali-treated EV, hemolymph-derived NOV, intracellular NOV from culture cells, and intracellular NOV from the fat body (Volkman et al., 1976).

Figure 21 shows the comparative curves of anti-AcMNPV LOVAL serum and anti-AcMNPV EV serum versus AcMNPV LOVAL. The curves of the untreated sera were very nearly identical, indicating that the neutralization titer in the heterologous serum was equal to that of the homologous serum, but when the sera were heat inactivated, the 50% neutralization titer of the homologous antisera (anti-AcMNPV LOVAL) was apparently increased (about four-fold), whereas the titer of the heterologous antisera appeared to be relatively unchanged.

Figure 22 depicts the neutralization capability of anti-AcMNPV EV serum (heat inactivated and untreated) versus AcMNPV EV, and the untreated serum versus both LOVAL and EV of RoMNPV. It should be noted that the

titer of this serum not only was identical with the comparable forms of AcMNPV and RoMNPV, but was higher against the NOV than against the alkali-liberated viruses.

Neutralization of intracellular NOV from both TN-368-10 cells and the fat body of T. ni larvae with heat-inactivated anti-AcMNPV EV (Figure 22) indicated that the intracellular NOV shared a biologically important antigenic determinant(s) with EV that was not present or accessible on LOVAL.

Figure 24 shows the results of the neutralization of both AcMNPV EV and LOVAL with AcMNPV LOVAL-adsorbed anti-AcMNPV EV. As can be seen, there was no remaining neutralization activity against LOVAL, whereas there was no change in the titer against the EV. These results confirm the conclusion that the LOVAL EV forms are antigenically distinguishable.

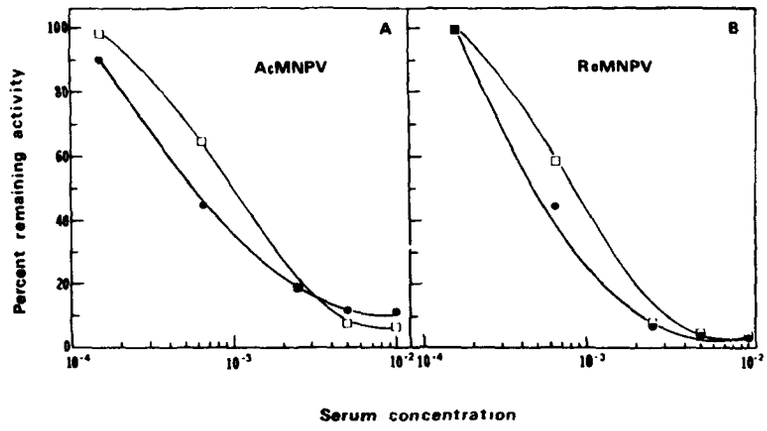

Figure 20. Neutralization of AcMNPV LOVAL (A) and RoMNPV LOVAL (B) with rabbit antisera generated against AcMNPV LOVAL (□) and RoMNPV LOVAL (●). Virus samples were incubated for one hr at 37°C with the indicated concentration of antiserum, and then 0.1 ml was assayed for biological activity. (The concentration of undiluted antiserum is 1). The 100% value of biological activity was the average of the control virus samples incubated with preimmune serum at the various concentrations. All assays were done in duplicate.

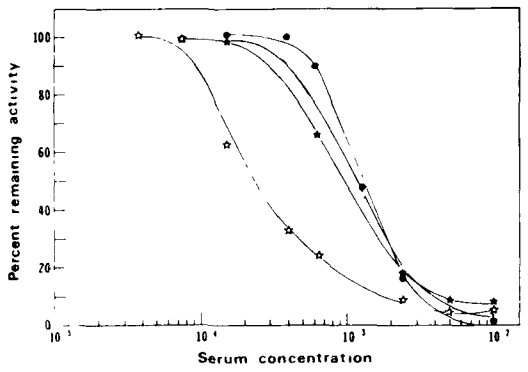

Figure 21. Neutralization of AcMNPV LOVAL with heat inactivated (☆) and untreated (★) anti-AcMNPV LOVAL serum, and with heat-inactivated (O) and untreated (●) anti-AcMNPV EV serum. Neutralization was carried out as described in Fig. 4 and the test. Heat inactivation of antisera is described in the test.

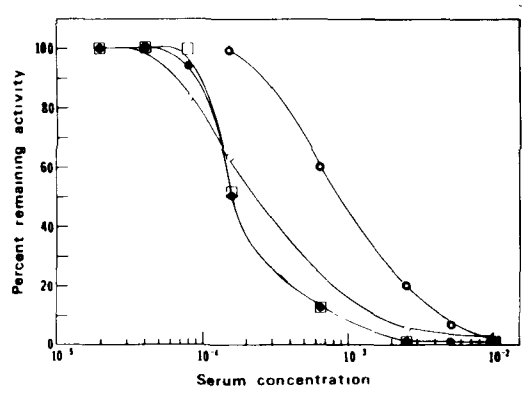

Figure 22. Virus neutralization with untreated anti-AcMNPV EV serum of AcMNPV PMB-NOV (●), RoMNPV EV (O), and RoMNPV LOVAL ◐. Heat inactivation of this antiserum did not alter the neutralization curve of AcMNPV EV (□).

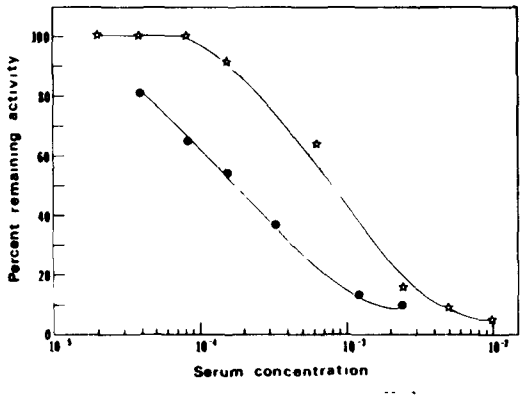

Figure 23. Neutralization of intracellular nonoccluded virus from both TN-368-13 cells (●) and the fat body of infected T. ni larvae (☆) with heat-inactivated anti-AcMNPV EV serum.

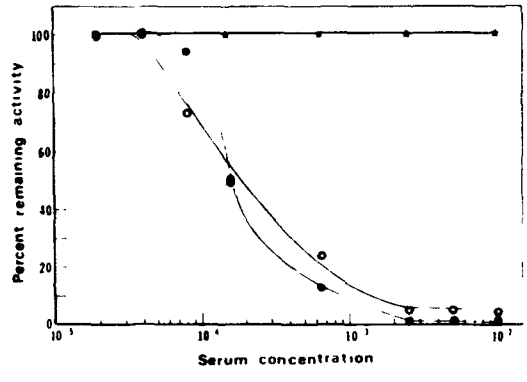

Figure 24. AcMNPV LOVAL-adsorbed anti-AcMNPV EV serum versus AcMNPV EV ✪ and LOVAL (★). The unadsorbed anti-AcMNPV EV versus AcMNPV EV curve is shown in comparison (●).

Discussion

The results of the neutralization tests showed that anti-RoMNPV and anti-AcMNPV LOVAL sera neutralize both the homologous and heterologous LOVAL forms of the virus, but neither will neutralize even the homologous nonoccluded forms of the virus. These results suggest that antigens important in biological activity are shared in the in vitro infectious LOVAL of two different viruses, but either are not present or are not exposed in the infectious forms of the NOV, or are not important in the expression of their biological activity. In any case, the two forms of in vitro infectious virus appear to be antigenically if not biologically different, whereas the same forms of two different viruses, AcMNPV and RoMNPV, share biologically important antigenic determinants.

The reciprocal tests, i.e., neutralization of the various forms with anti-AcMNPV EV, showed that all forms tested of both AcMNPV and RoMNPV could be neutralized, but that the titer was generally higher against the nonoccluded forms than the alkaline-liberated forms. Adsorption of anti-AcMNPV EV with AcMNPV LOVAL completely removed all neutralization activity against AcMNPV LOVAL, whereas the titer against AcMNPV EV and other nonoccluded forms was undiminished. These results confirm the initial findings that those antigens important in the neutralization of LOVAL and EV are different. They also indicate that whereas LOVAL does not possess an accessible antigen similar to the ones important for the neutralization of EV, EV does share the antigen that is active in the neutralization of LOVAL.

The results also indicated that antigens important in the neutralization of NOV other than EV are present and antigenic on EV. That the same antigen is active in the neutralization of all forms of NOV, however, has not been determined. The enhancement of the neutralization titer of untreated AcMNPV EV antisera against alternate nonoccluded forms by its adsorption with AcMNPV LOVAL indicates that the LOVAL is removing some component(s) of the serum that interferes with the neutralization of the alternate nonoccluded forms. This same component(s) does not interfere with the neutralization of EV, however. That this enhancement is also achieved by heat inactivation of the serum, and that heat inactivation and adsorption both does not have an additive effect, indicates that the component(s) is a nonspecific heat-labile factor.

The neutralization results presented in this report show rather significant antigenic differences between LOVAL, EV, and other NOV, and subtle differences between EV and othe NOV. These antigenic differences could very possibly be attributable to envelope differences. The results presented in this paper show that there are significant differences between the alkali-liberated and nonoccluded forms of the NPVs of R. ou and A. californica with regard to infectivity and antigenicity. These results, along with the mounting data indicating significant biochemical, biophysical, and morphological differences between the alkali-liberated and nonoccluded virions suggest structural differences that are indicative of differences in specificity, function, and role in the continuance of the infection cycle.

G. Preliminary Studies on Phenotypic and Genotypic Variation and/or Stability of Wild Viral Isolates After Passage Through Alternate Host Systems

In this portion of our studies we compared the stability and reproducibility of Autographa californica MNPV polyhedrin and virus structural polypeptides after passage through alternate host systems to the polypeptides of this virus form several wild isolates (Maruniak et al., 1978). A biochemical and biophysical comparison of these proteins from in vivo and in vitro sources will help in determining if changes occur which could involve host cell modifications, mutation, or recombination.

No differences in mobility of polyhedrin from the different sources can be detected on SDS polyacrylamide slab gels. When solubilized in alkali at 37°C for 1 and 2 h polyhedrin from larvae was degraded by the inclusion associated protease, while polyhedrin which is derived from infected tissue culture cells remains intact thus indicating lack of or diminished proteolytic activity.

Tryptic digests of polyhedrin purified from all sources utilized in this study showed little if any difference from the peptide maps. Keeping in mind that this technique will discriminate single amino acid differences in peptides, the polyhedrins were found to be very similar if not identical.

Using the improved technique of automated Edman degradation, the N-terminus of purified, undegraded 30,000 M.W. polyhedrin of Autographa MNPV purified from A. californica and T. ni larvae was determined as proline. In contrast, when polyhedrin was dansylated using the dansyl chloride technique, a weak orange flourescence near dansyl-glx appeared.

Although polyhedrin primary structure was not significantly different as determined by peptide mapping and amino acid analysis, minor quantitative differences in AcMNPV virus structural polypeptides were found when comparing it from one in vitro and four in vivo sources by SDS-PAGE (Figure 25). As accurately as possible to 30 µg of each was loaded on gels, yet obvious quantitative differences appeared especially with VP68 (Virus Protein), VP33, VP22, VP18, and VP16. There appeared to be more of VP68 in AcMNPV isolate D (Falcon) while there was significantly less in isolate B (Ruud). A difference in VP32 was noted by more of this protein in isolate C obtained from Vail. More of VP22 was found in that from isolate D (Falcon), while there was much less of the protein if any in AcMNPV isolate B (Ruud). The lower molecular weight protein, VP18, was reduced in amount in our AcMNPV isolate E. Finally, a quantitative difference appeared in VP16 being diminished in AcMNPV isolate D (Falcon) and also diminished in our preparation of enveloped nucleocapsids, F, isolated from tissue culture derived polyhedra. Some qualitative differences noted were VP22 being apparently absent or diminished compared to the amount loaded on the gel in the AcMNPV preparation from L. Ruud (B) and perhaps the presence of the diffuse VP18.5 from the AcMNPV prepared in this laboratory (E). It should be noted that the polypeptides of enveloped nucleocapsids derived from tissue culture polyhedra closely paralleled those for the same isolate derived from larvae.

It was shown in one of our studies (Maruniak and Summers, 1978) that T. ni granulosis virus, T. ni nuclear polyhedrosis virus, Autographa californica (AcMNPV), and Rachiplusia ou RoMNPV polyhedrin and granulin primary sequences were specific for each virus. Using the same biochemical techniques, we could not detect any alteration in primary sequence of AcMNPV polyhedrin after passage through alternate host systems. It should be noted that AcMNPV from this laboratory has been propagated 20 to 30 times in T. ni larvae and likewise, AcMNPV extracellular virus which was used to infect tissue culture cells has been cultured many times through the cells since 1975. This data supports the hypothesis that polyhedrin is a virus specific protein, and by this method of evaluation appears to remain stable upon continuous passage through alternate biological systems.

R. J. Cibulsky, et al., (1977) also found peptide maps of polyhedrin from AcMNPV from A. californica and T. ni larvae to be indistinguishable. However, we cannot directly compare our results due to: (1) the use of a different system for electrophoresis; (2) the virus associated protease was not inactivated in that study; and, (3) peptide mapping was carried out on a protein preparation with bands of several molecular weights. It has been discussed previously in this report that an active virus protease and a heterogenous protein can cause artifacts in peptide mapping, and therefore, it is essential to inactivate the protease and provide evidence of purity of the polypeptide preparation. However, as compared to polyhedra from larvae, there appears to be little if any protease activity associated with tissue culture derived polyhedra when alkaline solubilized up to 2 hours at 37°C and, hence little or no degradation of polyhedrin. We therefore recommend that investigators consider tissue culture derived polyhedra as an additional source for undegraded polyhedrin.

Further consideration is being given to enveloped nucleocapsids showing AcMNPV derived from various sources, both in vivo and in vitro, exhibits very similar polypeptide profiles on SDS polyacrylamide slab gels. SDS-PAGE has been demonstrated to be a valid technique for showing differences in virus strains by Montcastle and Choppin (1977), among four measles virus strains and be Gupta et al., (1977) for several strains of human cytomegalovirus where some qualitative and quantitative differences are seen. Furthermore, we do note some quantitative and qualitative differences in virus structural proteins, for which the molecular basis of these differences and their functional significance remain to be elucidated, the major polypeptides were conserved in the viruses from the three laboratories as well as those collected from the field. We have also determined in studies we are continuing that plaque purified isolates show distinct phenotypic and genotypic differences existing within a wild isolate of AcMNPV.

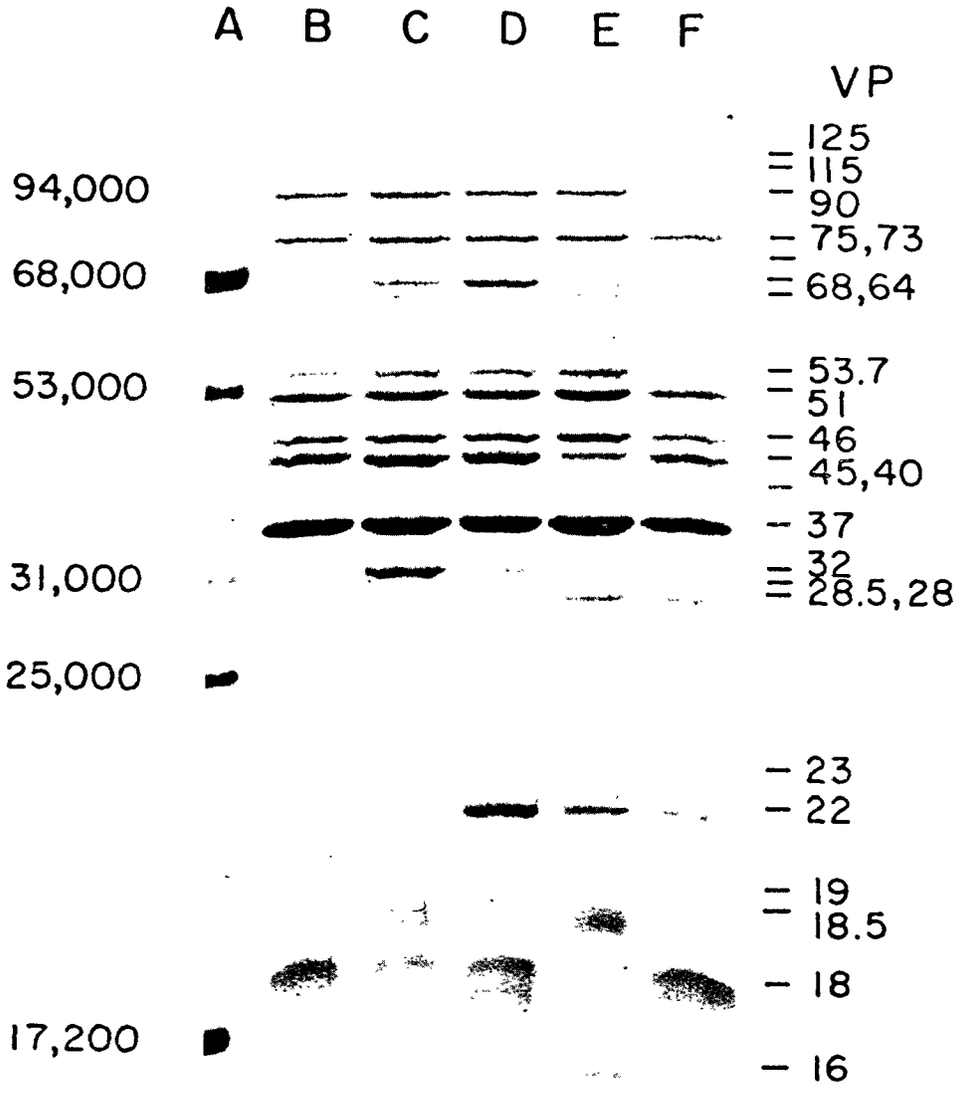

Figure 25. SDS-polyacrylamide gel electrophoresis of AcMNPV structural polypeptides. The SDS-gel composition was 10.8% acrylamide (Acrylamide/Bisacrylamide = 37.5/1). (A) Molecular weight standards are listed in Fig. 1. (B) AcMNPV from infected field insects from L. Ruud. (C) AcMNPV in Autographa californica obtained from P. Vail. (D) AcMNPV in T. ni obtained from L. Falcon. (E) AcMNPV in T. ni from this laboratory. (F) AcMNPV derived from TN-368-10 insect tissue culture cells in this laboratory.

H. Use of Restriction Enzymes for Evaluating the Genome Structure of Plaque-Purified and Wild Isolates of Baculovirus

The original source(s) of baculoviruses used in basic and field applied research or for commercial development has been from field-collected, infected insects subsequently propagated in the laboratory. For the characterization of wild isolate viruses, structural, immunological and biological criteria can be employed. A knowledge of the protein compositon and viral DNA structure would provide identifiable phenotypic and genotypic markers.

In order to identify baculoviruses, it is necessary to take into account the complex replication behavior they exhibit both in vivo and in cell culture, producing infectious forms with different biological and structural properties that have not yet been fully characterized. The nuclear polyhedrosis virus (NPV) of Autographa californica is known to produce at least three infectious forms: a) viruses occluded in the nucleus with a single nucleocapsid per envelope (singles); b) with multiple nucleocapsids per envelope (multiples); and c) extracellular virus that has budded from the plasma membrane (Summers and Volkman, 1976; Volkman et al., 1976). The structural polypeptide composition of AcMNPV singles and multiples, has been shown (Summers and Smith, 1978) to be both quantitatively and qualitatively different. Herein, we show that the structural polypeptides of extracellular AcMNPV exhibit different electrophoretic patterns in polyacrylamide when compared to singles or multiples. Polypeptide differences among these three infectious forms suggests the possibility of genetic variation. Therefore, we isolated the three forms from wild-type, AcMNPV to compare their viral DNAs using restriction endonuclease digestion and fragment analysis.

The uncertainty of the genetic purity and the possibility of genetic change (or contamination by other baculoviruses) during passage in laboratory insect colonies and in cell cultures emphasizes the need to determine the genomic heterogeneity and the genetic stability of wild baculovirus isolates. The NPV of A. californica was purified in cell culture by multiple plaque purifications and the viral DNAs compared using agarose electrophoresis of EcoR-1 and HindIII restriction endonuclease fragments. By comparing the restriction fragment patterns of each of the plaque-purified viral DNAs to the wild-type viral DNAs, we detected and quantified genetic variants existing in wild-type AcMNPV.

EcoR-1 Endonuclease Digests of Baculoviruses DNAs

The EcoR-1 restriction patterns of nine DNAs from A. californica MNPV (Ac), Rachiplusia ou MNPV (Ro), Trichoplusia ni SNPV (TnS), Porthetria dispar MNPV (Pd), Spodoptera exigua MNPV (Se), Heliothis zea SNPV (Hz), and Heliothis armigera MNPV (Ha), and from T. ni (TnG) and Spodoptera frugiperda (SfG) granulosis virus are shown in Figure 26 (Smith and Summers, 1978).

Each DNA restriction pattern was further analyzed by scanning densitometry and molarity of each fragment determined as described in Materials and Methods. Submolar restriction fragments could be detected in four wild-type baculovirus DNAs, AcMNPV, TnGV, SfGV, and HaMNPV.

Comparison of Wild-Type DNAs

Wild-type AcMNPV was obtained from three sources: 1) our laboratory where the virus was originally received from Dr. Pat Vail and subsequently grown for several generations over a period of 4 years in T. ni larvae; 2) field-collected virus obtained from Dr. Lou Falcon, University of California, Berkeley; and 3) an original isolate of AcMNPV from Dr. Pat Vail grown in A. californica larvae. Restriction fragment analysis using EcoR-1 (not shown) and HindIII endonucleases with viral DNA from these three isolates were identical.

A comparison of the DNA restriction patterns using EcoR-1 and HindIII endonucleases of three wild-type AcMNPV forms, extracellular virus (E), singles (S), and multiples (M), showed no detectable differences (data not shown). The EcoR-1 and HindIII DNA restriction patterns from wild-type AcMNPV grown in larvae (L) or cell culture (C) were also identical.

Restriction Analysis of Plaque-Purified Virus DNA

DNA was purified from 11 plaque-purified AcMNPV isolates. Of the 11 plaque-purified viruses, three were extracellular virus (E1, 2, 3) and four each from singles (S1, 2, 4, 5) and multiples (M1, 2, 3, 4). An analysis of each by EcoR-1 digestion gave a total of three restriction patterns designated as Types I, II, and III. Each type of AcMNPV had molecular weights of 79.0, 79.1, 81.0 megadaltons, respectively. The plaque-purified virus isolates E1, 2, and 3, S4, and 5, and M1, 2, and 4 were of Type I; S2 and M3 were of Type II; and only S1 was of the Type III EcoR-1 restriction pattern (Figure 27).

A feature common to uncloned AcMNPV DNAs, when cleaved with EcoR-1 and electrophoresed in 0.75 and 1.0% agarose is the presence of 23 restriction fragments. All but one of the fragments are major and in molar concentrations. A submolar fragment with a molecular weight of 2.5×10^6, which is missing. Type II DNA restriction pattern only in the absence of a fragment with a molecular weight of 2.4×10^6 and the presence of a 2.5×10^6 band as a major molar fragment. The EcoR-1 Type II DNA was identified in two of the eleven clones. This shows that uncloned AcMNPV DNA isolates contain detectable EcoR-1 Type I and II genomes. The EcoR-1 Type III restriction pattern is like the Type II except for a 7.0×10^6 fragment. This EcoR-1 Type III pattern was not detected in uncloned DNA. Inasmuch as only one of the 11 virus clones tested exhibited this pattern, it is likely a genomic variant in the wild isolate that is present in only a small percentage of the viral DNA population.

The restriction patterns of the 11 plaque-purified AcMNPVs produced by the HindIII enzyme showed that 8 of 11 viral DNAs were different. Digestion with HindIII gave more resolution and revealed differences in plaque-purified virus DNAs not detected with EcoR-1 digests. Consistent with the results of the EcoR-1 restriction enzyme analysis, HindIII pat-

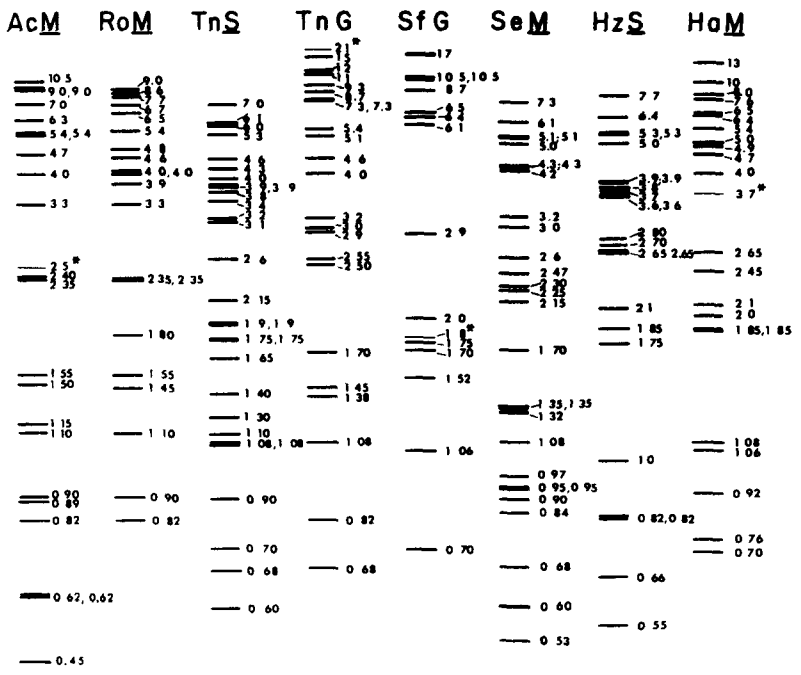

Figure 26. Schematic representation of the EcoR-1 restriction fragments for eight wild-type baculovirus DNAs. Molecular weights in megadaltons are given for each fragment and are the mean values from three separate agarose electrophoresis gels. The restriction pattern of PdMNPV DNA is not included as the co-migration of numerous high molecular weight EcoR-1 fragments made analysis difficult. Minor or submolar fragments have been indicated (*).

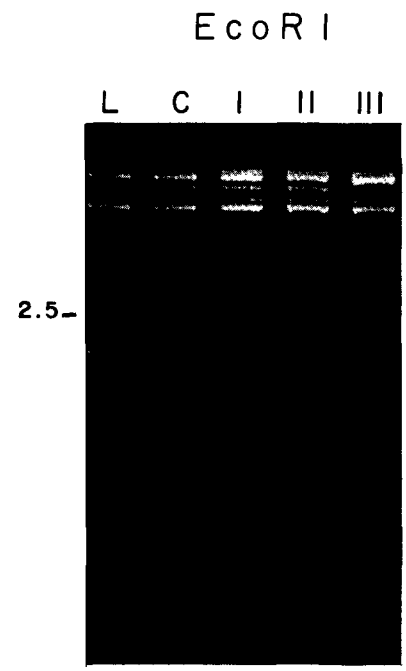

Figure 27. The DNA from plaque-purified AcMNPVs compared by electrophoresis of EcoR-1 restriction fragments as described in Fig. 2. The DNA restriction pattern from virus clones, E1, E2, E3, S4, S5, M1, M2, and M4 were Type I; S2 and M3 were Type II; and S1 was Type III. EcoR-1 restriction fragment of wild-type AcMNPV DNAs from infected insect larvae (L) and infected all culture (C) are shown. A minor fragment present wild-type DNA with a molecular weight of 2.5 x 10^6 daltons has been indicated.

terns of uncloned AcMNPV DNAs also contained several minor fragments. These minor fragments have mobilities that are the same as major, molar fragments found in several of the plaque-purified viral DNAs.

A comparison of the EcoR-1 restriction patterns of the DNAs from nine wild isolates of baculoviruses shows that this method is specific for virus genome identification (Figure 26). Only two of the baculovirus DNAs that were analyzed, AcMNPV and RoMNPV, showed similarity in restriction fragments, of which about 40% of the EcoR-1 and HindIII fragments co-migrated. The EcoR-1 restriction patterns of DNA from Orgya pseudotsugat SNPV and MNPV (Rohrman et al., 1978) also appear to be unique in comparison with the DNAs analyzed in this report.

Each infectious form of AcMNPV whether derived from in vitro, extracellular virus, or in vivo, singles or multiples from polyhedra (Summers and Smith, 1978), have certain structural polypeptides which differ quantitatively and qualitatively. These infectious forms are apparently phenotypes inasmuch as 1) DNA purified from wild-type AcMNPV extracellular virus or singles and multiples from polyhedra has similar restriction patterns, and 2) restriction enzyme digests of plaque-purified isolates for each infectious type revealed the presence of genetic variants; however, it was not possible to correlate a given restriction pattern with any of the three infectious forms. It is possible that minor genomic variants, not detected with EcoR-1 or HindIII restriction enzymes, could be responsible for the observed differences, but we presently feel this is unlikely. Furthermore, extracellular virus is 2000-fold more infectious than singles or multiples derived from polyhedra, yet no difference was detected in plaque-purified isolates from the three infectious forms which would account for a more efficient variant (Volkman et al., 1977). The use of other restriction enzymes and molecular hybridization will be needed to additionally confirm that these distinctly different viral forms do have the same genome.

The DNA restriction patterns of wild-type AcMNPV, TnGV, SfGV, and HaMNPV consistently showed submolar fragments. These fragments could not be attributed to incomplete digestion products since concentration did not change the pattern. The presence of submolar fragments suggests that intermolecular heterogeneity is present in these wild-type virus DNAs. Variation in the viral DNA molecules could be of two types: 1) molecules with the same overall base and gene composition but with a rearrangement of certain genome segments, for example, inversion of specific regions detected in herpes virus DNA (Hayward et al., 1975); or 2) molecules with different gene composition as would result from recombination, site-specific mutations, or contamination with host or other virus DNAs.

The nature of the genomic variation found in wild-type baculovirus DNAs, as evidenced by the presence of submolar fragments, is not known. However, the restriction enzyme patterns of DNA from plaque-purified AcMNPV suggests that heterogeneity is not due to contamination of other viral DNA(s) but to a mixture of AcMNPV genetic variants. This is because the submolar restriction fragments found in wild-type AcMNPV DNA had the same mobility as molar fragments in the restriction patterns of certain cloned AcMNPV DNAs.

Of 11 plaque-purified AcMNPVs compared in this study, eight had different EcoR-1 and/or HindIII restriction patterns. With certain of the plaque-purified viruses, no change was detected in viral DNA restriction endonuclease fragment patterns upon either additional plaque purification or undiluted serial passage 16 times through cell culture. This provides additional evidence that AcMNPV variants are a natural occurrence and are not induced by laboratory manipulation.

The DNAs of wild-type AcMNPV from two other sources when analyzed with EcoR-1 and HindIII and compared to DNA of AcMNPV grown in this laboratory, had identical restriction patterns with respect to both major and minor fragments. This suggests that these sources of AcMNPV are also genetically heterogenous and may contain some of the same variants. Plaque purification of a wild-type baculovirus, should it prove to be concomitant with biological or phenotypic changes, will have significant implications as to the specificity and any potential for altered host range.

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16. ABSTRACT

Biological pesticides, in particular the microbial formulations, are fundamentally different from chemical pesticides in the nature and mode of action of the active agent. The pesticidal action is dependent on the activities of living organisms. Identification, detection and monitoring methods for biological pesticides, because of their nature and characteristics, are divergent from those classically associated with chemical toxicants. Therefore, a new class of standardized, specific and sensitive methods must be developed.

One objective of this grant was the development, adapttation and application of specific sensitive diagnostic and clinical techniques for identification, detection and monitoring of viral pesticides. A portion of this research was also commited to study some of the basic biology and characteristics of baculoviruses so that a more thorough understanding of the limitations of the developed monitoring technology would be better understood. This technology can now be applied for the assessment of health and ecological effects, as well as for regulatory concerns.

17. KEY WORDS AND DOCUMENT ANALYSIS

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