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CHARACTERIZATION OF SHRIMP BACULOVIRUS

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CHARACTERIZATION OF SHRIMP BACULOVIRUS

by

Max D. Summers
The Cell Research Institute
and
Department of Botany
The University of Texas at Austin
Austin, Texas 78712

EPA Grant R-803395

Project Officer

John A. Couch
Gulf Breeze Environmental Research Laboratory
Gulf Breeze, Florida 32561

Environmental Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Gulf Breeze, Florida 32561

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FOREWORD

The protection of our aquatic resources from damage caused by chemical or biological pesticidal agents requires that regulations controlling the use of specific agents in pest control be formulated on a sound scientific basis. Accurate information on the novel biological control agents and related biological entities being developed for use as pesticides is needed to insure their efficacy and safety. The Environmental Research Laboratory, Gulf Breeze, contributes to this need through research aimed at determining precise identification, isolation, and characterization of biological control agents and related species found naturally in aquatic organisms. The recent finding by Gulf Breeze researchers of the first Baculovirus naturally occurring in commercial shrimp illustrates the need to better understand the nature of this large assemblage of arthropod viruses, some of which are under development as biological control agents.

Thomas W. Duke
Laboratory Director

ABSTRACT

The research undertaken involved the partial characterization of a baculovirus of the pink shrimp, Penaeus duorarum. The significance of the study is related to the fact that the shrimp baculovirus is morphologically similar to insect baculoviruses which were considered unique to insect arthropods prior to the discovery of shrimp nuclear polyhedrosis baculovirus (NPV). Further, insect baculoviruses are being developed and applied as microbial pesticides for the control of certain agricultural insect pests. Whereas the baculovirus diseases in pests of agricultural or medical importance are considered a desirable relationship, a baculovirus infection in shrimp is an undesirable one.

Research included investigations of the biochemical, structural, and, where appropriate, biological properties of the shrimp virus as compared to those of known and characterized properties of insect baculoviruses, both granulosis and NPVs.

Evidence for any structural relatedness of the shrimp NPV to insect NPVs has been confirmed in cross-reactions of purified shrimp NPV polyhedrin and infected shrimp tissues to insect baculovirus antisera.

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ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

AcMNPV	-- *Autographa californica* nuclear polyhedrosis virus
BSA	-- bovine serum albumin
ccDNA	-- covalently closed DNA
CPE	-- cytopathic effect
DAS	-- dilute alkaline saline, pH 10.9
dlDNA	-- double-stranded linear DNA
DNA	-- deoxyribonucleic acid
ETDA	-- ethylenediamine tetraacetic acid
GV	-- granulosis virus
µg	-- microgram
mg	-- milligram
ml	-- milliliter
mM	-- millimolar
NP-40	-- nonident 40
NPV	-- nuclear polyhedrosis virus
PdSNPV	-- *Penaeus duorarum* nuclear polyhedrosis virus
rcDNA	-- relaxed circular DNA
RIA	-- radioimmunoassay
rpm	-- revolutions per minute
SDS	-- sodium dodecyl sulfate
SDS-PAGE	-- sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SSC	-- standard saline citrate
TN-368	-- *Trichoplusia ni* 368 cells
TN-368-10	-- Clone 10, *Trichoplusia ni* cells
UV	-- ultraviolet

SYMBOLS

Å	-- Angstroms
C	-- Celsius
hr	-- hour
M	-- many nucleocapsids per envelope
S	-- enveloped single nucleocapsid

SECTION 1

INTRODUCTION

Enveloped rod-shaped DNA viruses which are occluded in proteinic crystals have been discovered and described as viruses unique for insect arthropods. Routinely these viruses are described as nuclear polyhedrosis (NPV) and granulosis viruses (GV) (Wildy, 1971) and more recently reclassified in Baculoviridae. Baculovirus cytopathology and ultrastructure have been studied extensively and until recently it was considered that they were unique in that they were the only viruses known to be occluded and to be specific for insect hosts. Now it is known that this is not the case (Volkman et al., 1976). Recent reports have shown that occlusion does not necessarily occur for a baculovirus during all stages of the biological cycle. That is, it may exist in both the occluded and nonoccluded states during the infection sequence. The discovery of a baculovirus of the pink shrimp, Penaeus duorarum (Couch, 1974; Couch et al., 1975), has now extended the host range for this class of viruses into the class Crustacea. Another report of a possible baculovirus cites infection of the nuclei of hemocytes and connective tissue elements of the European crab Carcinus maenas (Bonami, 1976). More recently, a baculovirus of the blue crab Callinectes sapidus was discovered in the epithelium of hepatopancreatic cells (Johnson, 1976). The virus was observed in both juvenile and adult crabs of both sexes that had been collected in the Chincoteague Bay, Virginia, and in Chesapeake Bay, Maryland, and its tributaries. In one group of crabs sampled in the Chincoteague Bay, 52% were reported infected.

The significance of the observations of baculoviruses in noninsect arthropods leads to a great deal of curiosity about any relatedness to insect baculoviruses. This is especially significant from the standpoint of the use of insect baculoviruses as biological pesticides, a development which has recently resulted in the registration of two insect baculoviruses for control of insect pests. Furthermore, characterization of the shrimp baculovirus will aid in providing technology for monitoring in order to study any effect that the virus may have upon populations of shrimp in nature and the extent of prevalence. The latter is particularly important from the standpoint of understanding the direct effect of the virus on its natural host (Couch et al., 1975). There is direct evidence that severe cytopathological effects occur in hepatopancreatic cells of infected organisms. There is additional evidence that the virus may cause epizootic mortalities in feral shrimp and larval shrimp in culture. Further, it is important to understand what interactions may occur with stress involving chemical pollutants. Couch et al. (1975, 1976, 1977) reports that polychlorinated biphenyls may increase the prevalence of patent virus infections

in experimental animals. The role that pollutants may play as stressors can be effectively studied only through the use of quantitative and less ambiguous means of evaluating virus infections.

Lastly, it is important to study host specificity with regard to baculoviruses in more definitive detail. This is especially important relative to the proposed role and use of insect baculoviruses as pesticides. Although there appears to be little danger involved in the use of present baculoviruses as viral pesticides, the problem of host specificity will require a more comprehensive definition. The discovery of baculoviruses in marine crustaceans from different areas of the world suggests that a restricted virus in terms of specificity for only insect arthropods is not now as absolute as previously thought.

SECTION 2

CONCLUSIONS

Studying the shrimp baculovirus in its natural environment and attempting to recover sufficient quantities for characterization studies were difficult during these studies and definitely limited the magnitude of effort and the extent of the results obtained.

The shrimp baculovirus has chemical and physical properties which, although similar to insect baculoviruses, are distinctly different. One of the major problems was our inability to use routine dilute alkaline saline solubilization in order to disaggregate the shrimp polyhedrin from its crystalline form, and at the same time release enveloped nucleocapsids. Ionic and nonionic detergents were required for the crystal solubilization process and thereby the physical integrity of the enveloped nucleocapsid was destroyed.

Attempts to recover enveloped nucleocapsids directly from infected shrimp tissue were only partially successful in that only very limited amounts of virus could be recovered after isopycnic banding on sucrose gradients. Therefore, attempts to continue work with that form of the virus for comparison with insect baculoviruses were not continued.

When comparing enveloped nucleocapsid structure and occlusion in a protein crystal, the general ultrastructural relationships are similar to those routinely observed for insect baculoviruses. However, there are some dissimilarities. The lattice spacing, and therefore the unit structure of the crystal, is significantly larger and the polyhedral membrane is absent.

Since enveloped nucleocapsids could not be recovered in sufficient quantities, no structural studies were done on that form of the virus. However, SDS-PAGE of shrimp polyhedrin demonstrated that it was significantly larger as compared with insect baculovirus granulins and polyhedrins. On the surface this might be considered evidence of unrelatedness since the insect baculovirus polyhedrins and granulins have shown remarkable similarity in size and composition. Summers and Smith (1976) demonstrated by two-dimensional high voltage electrophoresis and peptide mapping that, although highly purified insect baculovirus polyhedrins and granulins demonstrated some similar peptides and therefore similar primary structures, there were different peptides indicating that each protein (in association with its enveloped nucleocapsid component) was specific for that virus.

Serological studies published to date have revealed cross-reactions of antisera and antigens for this class of baculovirus proteins. Initial

attempts to compare shrimp polyhedrin by immunodiffusion were negative; therefore, we considered that the protein was not related to the insect baculovirus structural proteins. However, denaturing agents, such as SDS used in the appropriate concentration so as not to introduce artifact, have opened the conformation of the shrimp polyhedrin and revealed some cross-reaction with insect baculovirus polyhedrin antisera. In particular, a cross-reaction with AcMNPV polyhedrin and TnGV granulin and antisera was significant and confirms that similar primary sequence or antigenic sites do exist in shrimp and insect virus polyhedrins.

These observations may lead to considerable speculation concerning the evolution of this class of unique proteins in association with virus infections. The nature and the possibility of this relatedness, however, should not be considered a serious topic for speculation until more complete studies have been conducted.

The shrimp baculovirus DNA, in terms of its structural properties and size, very definitely is similar to insect baculovirus DNAs. The relative ratio of ccDNA relative to rcDNA and dlDNA are remarkably similar. However, additional information on relatedness cannot be provided at this time because physical properties or information such as base-ratio analyses are not adequate. At first it was not thought possible to utilize any one technique for comparing the shrimp DNA with other baculovirus DNAs because only very small quantities could be obtained. With improved use and application of ^{125}I-labeling of nucleic acids, it is now possible to conduct physical mapping experiments with restriction endonuclease enzymes. Gene segments with similar base sequences could or should be revealed by this technique; such studies are contemplated for future investigations.

The preliminary studies in invertebrate cell culture were entirely inadequate and were merely probes or superficial attempts to see if the shrimp virus could be induced to replicate in vitro. However, all of those studies are now difficult to evaluate because it is known that polyhedra formation is not considered a reliable indicator of virus replication. It is possible in certain cell lines that virus replication could have occurred, but was not detectable by visual observation on the phase or electron microscopes. The development and use of shrimp baculovirus antisera for both polyhedrin and infected tissue will enable continuation of our work with more confidence and definitive applications. Immune peroxidase has been developed and is now being routinely used for both insect baculovirus polyhedrins and enveloped nucleocapsid detection in this laboratory (Summers and Hsieh, manuscript in preparation). It is known to be a reliable and semi-quantitative technique. As soon as we obtain the shrimp virus antiserum, we will develop a similar technology and application.

The serological relatedness of shrimp and insect baculovirus polyhedrins is of considerable importance and the most positive aspect of this research project. It demonstrates that insect and shrimp viral proteins do have a similar antigen or antigenic determinants which are recognized by insect baculovirus antisera. This has been confirmed in two kinds of serological assays: immunodiffusion, which is good for specificity but weak in terms of sensitivity, and radioimmunoassay (RIA), which is a very sensitive

quantitative and specific assay. Both serological techniques are being utilized for insect baculovirus detection and identification in this laboratory (Summers and Hoops, manuscript in preparation; Ohba et al., manuscript in preparation). Furthermore, this technique has been shown to be highly reliable. When shrimp virus antisera are available, we will attempt to adapt RIA to that system as well. Therefore, if appropriate financial support is available it will be possible to use three major serological techniques: immunodiffusion, RIA, and immunoperoxidase. The complete capability for shrimp baculovirus detection, identification, monitoring, and screening will be available on both a quantitative and qualitative basis.

SECTION 3

RECOMMENDATIONS

Attempts to develop *in vitro* systems for the replication and production of shrimp baculovirus is highly desirable; however, it will require an empirical approach and very likely will be a long-term effort. Many different cell lines, conditions, and temperatures should be utilized in any attempt to introduce the virus into an *in vitro* system. Furthermore, such studies should not be conducted until a reliable screening procedure is available for the detection of both polyhedrin production and enveloped nucleocapsid replication as discussed earlier. Baculoviruses are now known to replicate in the absence of polyhedra formation and therefore the absence of the crystalline structure does not ensure that virus replication is not occurring. It is not practical to screen a variety of cell lines and/or experimental conditions for virus replication by electron microscopy. The immunoperoxidase technique is easily and reliably adapted for this kind of screening procedure.

The study of serological relatedness of the shrimp baculovirus to insect baculoviruses should be continued. With anticipated financial support, this work will be continued in this laboratory as soon as the appropriate antisera are available. Our inability to obtain a serological cross-reaction with enveloped nucleocapsids is very likely due to the lack of sufficient concentrations of virus to detect such cross-reactions. More tissue and virus will be required for such studies. Regardless, as stated earlier, the highest priority research should be the development of RIA and immunoperoxidase serological techniques in order to have available quantitative and qualitative detection and monitoring technology.

Although academic at this time, continued studies of the shrimp DNA should be encouraged, especially in the area of physical mapping of virus genomes, by use of specific restriction endonuclease enzymes and hybridization of apparently similar fragments.

SECTION 4

MATERIALS AND METHODS

SELECTION AND COLLECTION OF INFECTED ORGANISMS

Pink shrimp (Penaeus duorarum) were screened for tissues infected with shrimp baculovirus (P. duorarum NPV = PdSNPV) at Gulf Breeze, Florida. The availability of infected shrimp in large numbers was sporadic, and occasionally natural dips occurred in the prevalence when infected material was not available. The unavailability of shrimp on occasion was probably due to several possibilities: 1) The shrimp obtained were not always from the epicenter of the infected population, and fluctuations of infectivity from the main source affected the availability; 2) The presence of an occasional red tide in the general areas where the shrimp were obtained may have resulted in the death of many patently infected organisms; 3) The shrimp available perhaps were not from the area the supplier designated. Shrimp were also selected from a fish market as an alternative to live shrimp; that approach was not fruitful.

The data in Table 1 for the period of November 5 to December 5, 1974, are representative of the magnitude of screening involved and the success obtained in acquiring infected tissue for one shipment. All tissue was kept at -90°C after dissection of the infected tissue from the organism and shipped to Austin, Texas, in dry ice. All infected material was maintained at -90°C before and after virus purification.

PURIFICATION OF OCCLUDED PdSNPV

NPV-infected shrimp hepatopancreatic tissue was homogenized in 0.2 M Tris-HCl containing 10^{-2} M EDTA, pH 7.8 (Tris buffer solution). The homogenate was immediately centrifuged at 2,000 rpm at 5°C for two minutes with the J-21 rotor and Beckman J-20 centrifuge. The pellet showed the presence of a large number of characteristically occluded viruses as visualized with brightfield and phase contrast light microscopy (Couch, 1974; Couch et al., 1975). After low speed centrifugation, no visible polyhedra remained in the supernatant. The supernatant was stored at -90°C, and the 2,000 rpm (480 x g) pellet resuspended by vortex mixing. One-half ml was layered on linear sucrose gradients, ranging from 40 to 60% (wt %), and centrifuged for 20 minutes at 33,000 rpm with the SW-41 rotor. Most of the pink color in the virus pellet remained at the top of the gradient. The occluded virus banded at approximately one-half the distance in the gradient.

TABLE 1. SCREENING AND COLLECTION OF INFECTED SHRIMP FROM 11/5 TO 12/5, 1974

Date sample completed	Number shrimp in sample	Number found infected	% infected	Shrimp type
11/5	190	25	13.1	Live pinks
11/7	80	2	2.5	Live pinks
11/22	225	0	0	Live pinks
11/30	210	0	0	Live browns
12/4	200	0	0	Live pinks
Total	905	27	3.0	

Although purification was achieved by a single centrifugation, the occluded virus band was removed from the sucrose gradient, pelleted by differential centrifugation, and then rebanded on another linear 40 to 60% sucrose gradient. The occluded virus was removed for protein and nucleic acid studies. The yield from a total of approximately 100 infected hepatopancreatic tissues ranging from lightly to heavily infected was about 2.4 mg, as estimated from protein analysis.

SOLUBILIZATION OF OCCLUDED VIRUS

Attempts were made to solubilize occluded shrimp virus by using the standard insect baculovirus technique. One-tenth ml of purified occluded shrimp virus (170 µg) was pelleted and washed with deionized water. To the pellet, dilute alkaline saline (DAS = 0.1 M sodium carbonate plus 0.05 M NaCl, pH 10.9) was added. Occluded *Autographa californica* NPV (AcMNPV) was treated similarly. After two hr of exposure to DAS at room temperature, no difference was observed in the light scattering of the shrimp baculovirus solution, suggesting that solubilization had not occurred. Observation on the phase microscope confirmed this. AcMNPV had been completely solubilized. At the end of two hr exposure in DAS at 37°C, the shrimp virus preparation was made 1% with respect to SDS. This was incubated for an additional one hr at 37°C when it was noted that no turbidity remained in the tube.

AMINO ACID ANALYSIS

One hundred fifty µg of the PdSNPV purified by isopycnic banding in sucrose gradients was pelleted, washed free of buffer solution, and resuspended

in 0.2 ml of 1 mM NH_4CHO_3 (pH 11). The preparation did not immediately show evidence of solubilization. Two-tenths ml of 15 M NH_4OH was added. After repeated heating at 70°C for two hr, solubilization was apparently achieved. The solubilized protein was dried under vacuum, and samples were hydrolyzed in aqueous HCl at 105°C for 24 and 48 hr (Summers and Smith, 1976). Values for threonine and serine were extrapolated to time zero.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Electrophoresis in a 10.8% polyacrylamide vertical gel slab in the presence of 0.1% SDS was done as described by Laemmli (1970). The samples were prepared for electrophoresis by the addition of 2% SDS and 5% mercaptoethanol in 0.25 M Tris (pH 6.8) with a final protein concentration of 1 mg/ml. The samples were heated at 100°C for three minutes and were subjected to electrophoresis for three hr at a constant power of 7.5 watts/gel slab. The gel slab was 14 x 12 cm and 1.5 mm thick. The gels were fixed and stained with 25% isopropanol, 10% acetic acid, and 0.04% Coomassie brilliant blue (R-250) for four hr and then destained in 10% acetic acid.

The method described by Weber and Osborn (1969) was used to determine the apparent molecular weights of the viral proteins as compared to the known weights of standard proteins.

PURIFICATION OF ENVELOPED NUCLEOCAPSIDS

Since it was not possible to recover enveloped nucleocapsids from DAS-solubilized occluded virus, an attempt was made to recover the non-occluded virus from the 2,000 rpm supernatant (see "Purfication of Occluded Virus") of homogenized tissue. A modification of the purification procedure for Oryctes NPV (Payne and Tinsley, 1974) was utilized. The 2,000 rpm supernatant in Tris buffer solution was centrifuged at 50,000 x g for 60 min with the SW-27 rotor. The supernatant was immediately stored at -90°C. One-half ml of the Tris buffer solution was added to the pellet and then allowed to set in the refrigerator overnight. The pellet was resuspended by vortex stirring and layered on top of a 25-50% (wt %) sucrose gradient constituted in the Tris buffer. The gradients were centrifuged at 100,000 x g for 90 min with the SW-41 rotor. The gradients also contained 1 ml of a 60% (wt %) sucrose cushion.

CELL CULTURE STUDIES

Homogenized PdSNPV infected hepatopancreatic tissue was added to each of the following cell lines in order to investigate ability to infect invertebrate cells: Trichoplusia ni (TN-368), Spodoptera frugiperda, Aedes albopictus, Culex salinarius, Armigeres subalbatus. The cell lines were maintained at 22°C and 28°C postinfection to see if temperature influenced susceptibility or replication.

DNA EXTRACTION

Occluded shrimp baculovirus was purified by equilibrium banding on sucrose gradients as described previously. Since it had been demonstrated that enveloped nucleocapsids could not be separated from the crystal protein by routine

DAS solubilization techniques, intact shrimp polyhedra were adjusted to a final concentration of 2% with respect to sodium lauryl-sarcosine in 1 x SSC (sodium saline citrate; 0.015 M sodium citrate, 0.15 M NaCl), 10 mM EDTA, pH 7.0 (Summers and Anderson, 1973). The sarcosyl treated polyhedral preparation was then heated at 60°C for 30 min. The solution cleared and was layered on top of a continuous gradient (density range 1.4 to 1.6 g/ml) ethidium bromide-cesium chloride (Summers and Anderson, 1973). Centrifugation was conducted for 24 hr at 40,000 rpm with the SW-65 rotor. After centrifugation, the gradients were visualized and photographed under ultraviolet light and the DNA bands recovered for Kleinschmidt preparations as described previously (Summers and Anderson, 1973; Summers, 1977).

DNA SPREADING AND ELECTRON MICROSCOPY

The Kleinschmidt spreading technique described by Lee, Davis, and Davison (1970) was utilized to visualize the circular forms of PdSNPV as well as estimate size. For estimates of size by measurements relative to calibrated standards, the Hitachi HU-11E electron microscope was used with a Fullam grid standard calibrated at 28,800 lines per inch. Also the density of DNA relative to DNA standards was estimated to be approximately 2×10^6 daltons per micron relative to purified T7 DNA bacteriphage standards.

IMMUNODIFFUSION

Double immunodiffusion was performed by a modification of the methods described by Oucterlony (1962). Briefly, 8 ml of 1% agarose in 0.01 M Tris buffer, pH 7.8, containing 0.01 M EDTA and 0.01% sodium azide was poured into disposable immunodiffusion plates (Miles Laboratories, Elkhart, IN), and wells 3 mm in diameter were cut around a 4-mm central well with a 4 mm center-to-center distance. The central well was filled with 20 µl of the appropriate undiluted polyhedrin antiserum. The peripheral wells were then filled with 10 µl of a 1 µg/µl solution of each antigen to be tested. The plates were placed in a humid atmosphere and incubated for 24 to 48 hr.

COMPETITION RADIOIMMUNOASSAY (RIA)

A modification of the micro solid-phase radioimmunoassay (RIA) techniques described by Purcell et al. (1973) was employed. Briefly, wells of a polystyrene microtiter plate (Limbro Scientific Co., New Haven, CT) were coated with 200 µl of a 0.02 M NaHCO buffer, pH 9.6, containing 25 µg/ml of Protein A (Pharmacia Fine Chemicals, Piscataway, NJ) and incubated for 18 hr at 4°C. The wells were aspirated and washed twice with 200 µl of 0.01 M Tris buffer, pH 7.8, containing M EDTA, 0.005 M KI, and 0.01% sodium azide. Two hundred ml of a 1:500 dilution of AcMNPV polyhedrin antiserum in Tris buffer was added to each well and incubated for 18 hr at 4°C. The wells were then aspirated, washed twice with Tris buffer, and coated with 200 µl of Tris buffer containing 5% BSA to prevent non-specific adsorption of antigens to unreacted sites on the wells. Following incubation for 4 hr at 4°C, the wells were washed twice with Tris buffer, and 10-fold concentrations of polyhedrins or granulins (0.01 to 1000 ng) were added and incubated for 18 hr at 4°C. The wells were then aspirated and

washed three times with Tris buffer. One nanogram of ^{125}I-AMN polyhedrin was then added to each well and incubated an additional 18 hr at 4°C. The wells were aspirated, washed three times with 5% BSA-Tris buffer, cut out, and counted for 1 min each in a gamma spectrophotometer (Searle, Model 1195, Arlington Heights, IL).

SECTION 5

RESULTS AND DISCUSSION

PURIFICATION OF POLYHEDRA

After banding in sucrose gradients, a very sharp light scattering zone was observed (Figure 1). Observations of a sample of the zone on the phase microscope revealed the presence of occluded virus.

The Tris-buffering solution did not appear to be a satisfactory or adequate medium in which to purify and maintain the occluded shrimp NPV. Periodic observations demonstrated that the crystals underwent fragmentation and/or disruption. Therefore, it was necessary to work quickly during the homogenizing and extraction procedure to obtain an optimal yield of occluded virus.

Subsequent storage of the purified occluded virus preparation also did not seem to stabilize the breakdown of the polyhedra. After a year of storage at -20°C, very few intact polyhedra could be observed by phase microscopy, even though the total protein concentration remained constant.

The addition of 0.1% SDS to the shrimp baculovirus in Tris buffer results in immediate solubilization of the polyhedra. A similar effect occurs by the addition of NP-40 at a final concentration of 1.0%. Unfortunately, enveloped nucleocapsids were not recovered from the preparation after this treatment.

The process of polyhedra dissociation as observed by phase microscopy is significantly different from that for AcMNPV. This was visualized by placing the baculovirus preparation on glass slides with a cover slip. One-tenth N NaOH was applied to the edge of the cover slide, and the sequential solubilization of the polyhedra was easily observed as the basic solution diffused beneath the cover slip. With AcMNPV, polyhedra solubilized immediately after exposure to the NaOH solution. The refractile polyhedra were observed to change to a dark color. However, the polyhedral "membrane" remained intact. The Brownian movement of bundles of enveloped nucleocapsids could be clearly observed inside the intact polyhedral membranes at high magnification on the phase microscope. In comparison, the shrimp baculovirus crystal began to fragment into smaller units, each of the smaller fragments undergoing subsequent breakdown until apparent solubilization occurred. This technique demonstrated that no polyhedral membrane could be observed in association with shrimp polyhedra. The presence of the polyhedral membrane associated with insect baculoviruses and the absence of such a structure on the shrimp NPV have been confirmed by electron

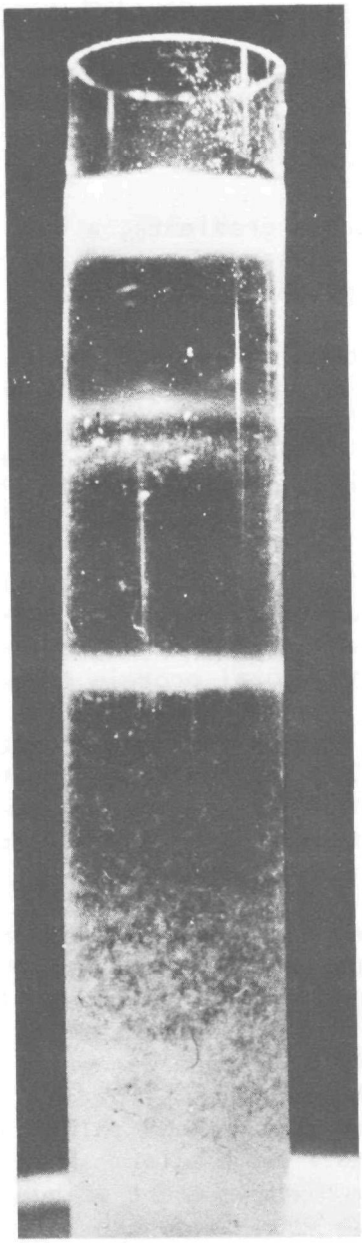

Figure 1. Isopycnic banding of shrimp baculovirus polyhedra in sucrose gradient. Density range: 1.1-1.3 g/ml. Conditions and details of the procedure are specified in "Materials and Methods."

microscopy (Summers and Arnott, 1969; Harrap, 1972; Couch, 1974).

For these studies it was not possible to isolate intact enveloped nucleocapsids from shrimp virus polyhedra; the routine DAS solubilization did not work and the use of ionic and nonionic detergents disrupted the physical integrity of the virus particle. Exposing the shrimp virus polyhedra to higher pH conditions may be effective. However, sufficient quantities of occluded virus were not available for continued experiments based on this approach. It was decided that serological studies would have higher priority and that continued studies attempting to isolate the enveloped nucleocapsids could be conducted later if enough material was available. This subject will be dealt with in the section on our attempts to extract enveloped nucleocapsids directly from infected hepatopancreatic tissue.

PURIFICATION OF ENVELOPED NUCLEOCAPSIDS FROM INFECTED TISSUE

As represented in Figure 2, four major zones were fractionated in sucrose gradients after centrifugation of the infected tissue homogenate. The top of the gradient where the sample was layered contained all of the pink color characteristic of the 50,000 x g pellet. The major band in the lower one-third to three-fourths region of the gradient was removed and only some unidentified material was observed by phase and electron microscopy. All four of these fractions were carefully removed by syringe, centrifuged at 50,000 x g for 60 min, and the pellets suspended in Tris buffer for observation on the electron microscope.

Enveloped nucleocapsids were observed only in the intermediate region, which showed some slight opaque or light refractile material but which did not exhibit any distinct bands. Since very few enveloped nucleocapsids were observed in this sample, it was not considered feasible to attempt to purify and recover enveloped nucleocapsids directly from homogenized tissue. Considerable quantities of tissue would be required and very likely are not available.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Shrimp polyhedrin prepared and maintained properly to minimize breakdown of the protein during storage and handling shows the presence of only one major band (Figure 3) with a molecular weight of 50,000. Polyhedrin handled frequently and at room temperature degrades. The molecular weight of 50,000 is distinctly different and much larger than that for insect baculovirus granulins and polyhedrins (Figure 3). Although Summers and Smith (1976) demonstrated that granulins and polyhedrins were approximately 28,000 daltons, the results reported here show that there is some variability in molecular weights of individual polyhedrins as measured by the improved SDS-PAGE technique. Of the granulins and polyhedrins shown, the molecular weights range from 25,500 to 31,000 daltons.

The size difference as measured by SDS-PAGE can be correlated with observations by electron microscopy (Summers and Arnott, 1971; Harrap, 1972; Couch, 1974). The crystal lattice spacing for most NPVs and GVs has

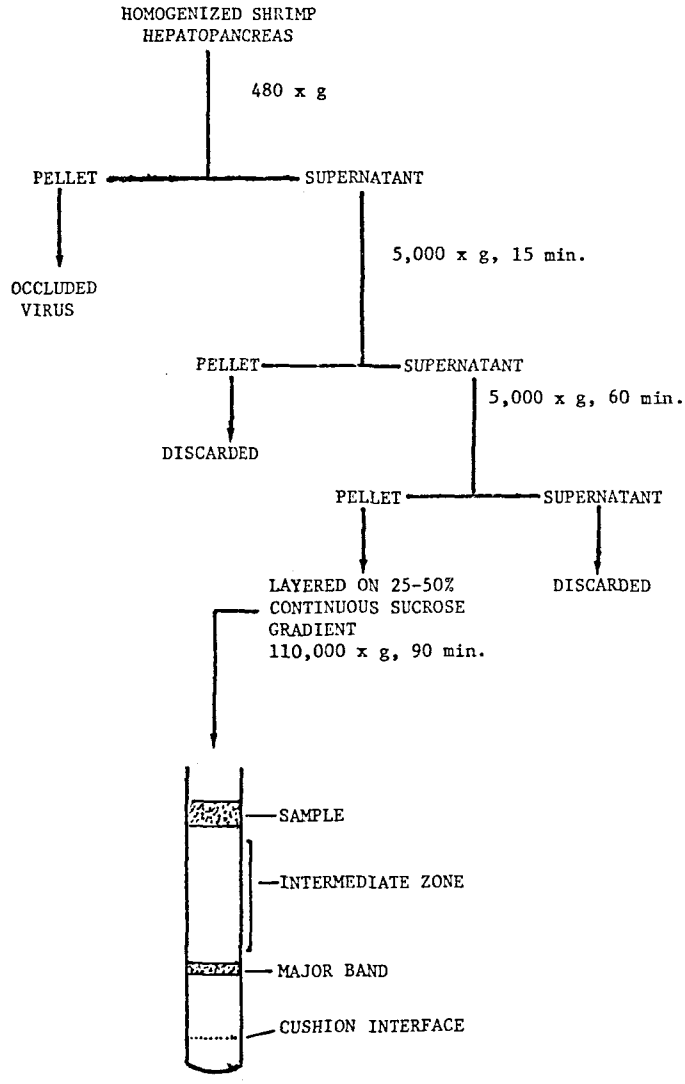

Figure 2

Figure 2. Purification schematic for fractionation of infected shrimp hepatopancreatic tissue.

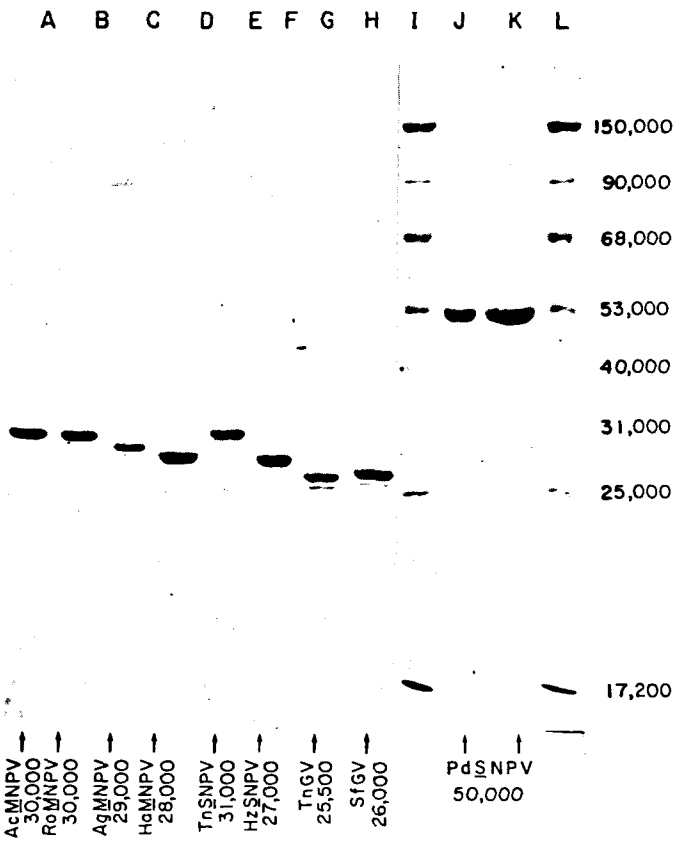

Figure 3. SDS-polyacrylamide gel electrophoresis of shrimp polyhedrin relative to insect baculovirus polyhedrins.

been measured to range from 40 to 70 Angstroms (Å). The subunit comprising the shrimp baculovirus crystal appears to be distinctly larger since the lattice space is approximately 120 Å.

AMINO ACID ANALYSIS

Amino acid analyses show (Table 2) that shrimp polyhedrin has approximately 471 residues based upon a molecular weight of 50,000 as determined by SDS-PAGE.

A comparison of the data in the table shows that insect baculoviruses exhibit remarkable similarity of amino acid composition. Although the shrimp polyhedrin also exhibits some general similar relationship, the shrimp polyhedrin does show distinct differences for certain amino acids: phenylalanine, tyrosine, isoleucine, methionine, glycine, and serine. Although in general amino acid analyses are usually not definitive as indicators of specific differences between or among proteins, data suggest some marked differences in amino acid sequences between the shrimp polyhedrin and those from insect baculoviruses. Consequently, it is not possible to discuss specific similarities or dissimilarities of the polyhedrins.

CELL CULTURE STUDIES

Although not given major emphasis at this time, preliminary attempts were made to infect invertebrate cell lines with homogenized heavily infected hepatopancreatic tissue.

Only Spodoptera frugiperda cells showed a cytopathic effect (CPE). Limited electron microscope observations of exposed S. frugiperda cells did not reveal any virus replication.

That study did not confirm whether the observed CPE was a result of some toxic effect of shrimp tissue or some effect of the virus. In an attempt to resolve this question, fractions from the gradient of fractionated viral material discussed previously were utilized for CPE studies. Spodoptera frugiperda cells were exposed to material from top, intermediate, major band, and cushion interface fractions. All preparations induced a CPE after three days' exposure. These observations indicated that the CPE was not a result of virus activity since apparently uninfected shrimp hepatopancreatic tissue also produced a similar CPE.

The CPE had the following characteristics: the cells exhibited some nuclear hypertrophy as well as the development of some refractile bodies in the cell. It was first thought that the refractile bodies were nuclear in origin. However, it is now believed that they were cytoplasmic after observations of the cells on the electron microscope. The size of the S. frugiperda cells made it difficult to differentiate the position of the refractile bodies in the cells. However, because of their size, refractile nature, and apparent position in the cells, it was easy to mistake the refractile bodies for proteinic crystals characteristic of insect baculovirus infections.

TABLE 2. AMINO ACID ANALYSIS OF GRANULINS AND POLYHEDRINS

Amino acid	Mole %						Residues					
	TnGV	SfGV	AcMNPV	TnSNPV	RoMNPV	PdSNPV	TnGV*	SfGV*	AcMNPV*	TnSNPV*	RoMNPV	PdSNPV
Asx	10.5	10.3	10.8	11.4	11.6	12.3	23.6	23.4	28.4	31.7	30.1	58
Thr	6.3	4.4	4.1	4.4	3.8	5.6	14.3	10.0	10.7	12.3	9.9	26
Ser	4.5	4.8	2.9	4.4	3.8	8.3	10.1	11.0	7.7	12.3	9.9	39
Glx	11.8	13.6	11.1	12.3	12.3	10.8	26.6	30.9	29.0	34.1	32.2	50
Pro	6.6	6.8	7.1	7.5	6.7	5.4	14.8	15.3	18.4	20.8	17.6	26
Gly	5.3	6.3	5.5	7.0	5.8	11.0	12.0	13.8	14.5	19.4	15.2	51
Ala	5.4	5.0	5.6	6.0	3.8	6.4	12.2	11.4	14.9	16.6	10.0	30
Val	7.0	5.7	8.1	7.6	8.2	7.5	15.7	12.9	21.1	21.1	21.4	35
1/2Cys	1.7	1.5	2.2	1.4	1.3	2.1	3.9	3.3	5.9	4.0	3.3	9
Met	0.4	0.5	0.4	0.4	0.3	1.5	0.91	1.2	1.1	1.1	0.6	7
Ileu	6.1	5.7	5.3	5.0	6.0	3.9	13.8	12.9	14.0	14.1	15.8	19
Leu	8.2	8.3	7.0	7.8	6.7	6.9	18.6	18.8	18.3	21.7	17.6	33
Tyr	3.2	4.1	4.9	4.0	5.0	0.2	7.2	9.4	12.8	11.2	13.1	1
Phe	6.7	4.1	5.2	3.7	4.9	1.2	15.2	9.2	13.7	10.4	12.8	5
Lys	5.5	6.6	8.6	8.2	8.8	8.9	12.4	15.0	22.4	22.8	22.8	42
His	3.4	2.5	2.3	2.3	2.4	3.3	7.7	5.7	6.1	6.3	6.2	16
Trp	3.2	3.7	2.9	2.0	2.5	---	3.3	9.2	7.5	5.5	6.5	---
Arg	6.0	6.3	6.1	5.0	6.0	5.0	13.5	14.3	15.9	13.8	15.8	24
Total							225.8	227.7	262.4	279.2	260.9	471
Molecular weight							25,500	26,000	30,000	31,000	30,000	50.000

* Summers and Smith, 1975

In order to conduct a more elaborate preliminary study the following experiment was conducted with TN-368-10, Culex tritaeniorhynchus, Spodoptera frugiperda, Armigeres subalbatus, and Culex salinarius cells. Each cell line was maintained at both 22°C and 28°C and exposed to homogenized infected hepatopancreatic tissue. In contrast to the first exposure period of only 48 hr, cells were prepared for electron microscopy after 2 and 4 days.

An extensive and comprehensive search of infected cells by electron microscopy was not conducted; however, preliminary observations of selected samples from those exposed cell lines did not show the presence of replicating virus. However, compared to the controls, exposed cells showed some distinct alteration of nuclear and cytoplasmic ultrastructure and some nuclear hypertrophy, as well as chromatin margination. It is difficult to evaluate the extent of nuclear membrane proliferation, although it did appear in a few observations. Since observations were made primarily for the purpose of detecting replicating virus, the differences between control and shrimp virus infected cells cannot be discussed here without a more extensive investigation.

PdSNPV DNA

Ethidium bromide-cesium chloride gradients containing shrimp baculovirus DNA exhibited the typical banding profile characteristic of a mixture of covalently closed DNA (ccDNA) and relaxed circular DNA (rcDNA) and double-stranded linear (dlDNA) molecules (Figure 4) (Summers and Anderson, 1973). The positions of the bands were not accurately measured, but with respect to the density of T4 DNA and the results of previous studies on insect baculovirus DNAs, the shrimp virus ccDNA banded at the characteristic density of 1.58 g/ml and the band characteristic of dlDNA and rcDNA banded at approximately 1.54 g/ml. The intensity of the bands under illumination by UV light revealed that the relative proportions of ccDNA at 1.58 g/ml to that of dlDNA and rcDNA at 1.54 g/ml was very similar to that of insect baculovirus DNA. Therefore, the yield of ccDNA was approximately 20 to 30% that of the total DNA preparation.

Since only limited quantities of the shrimp PdSNPV DNA were available (for example, the total amount in Figure 4 represents approximately 3 to 5 µg DNA), sedimentation studies for estimates of molecular weights were not feasible. Therefore, purified viral DNA recovered from the gradients shown in Figure 4 were prepared (Lee et al., 1970) for observation on the electron microscope.

The average mean molecular weight determined from several measurements on ten different molecules was 75×10^6 daltons $\pm 2 \times 10^6$. As can be seen from Figures 5-10 the characteristic conformation of a rcDNA is apparent. Figure 1 appears to be supercoiled DNA.

In a recent review on baculovirus DNAs (Summers, 1977), it has been shown (Table 3) that baculovirus DNAs range in size from 75 to 100 million daltons. Recent unpublished information (personal communication with J. Longworth) suggests that direct measurements on baculovirus genomes are going to be 10 to 20% less than molecular weights estimated by hydrodynamic

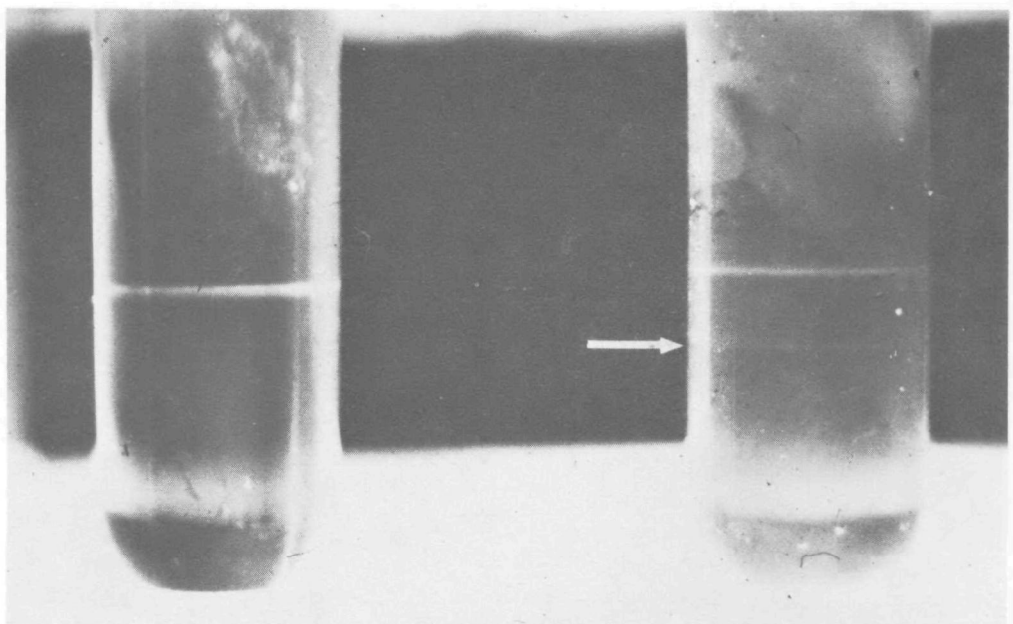

Figure 4. Equilibrium banding of shrimp baculovirus DNA in ethidium bromide-cesium chloride gradients. Methods and procedures according to Summers and Anderson (1973). Arrow designates covalently closed DNA (1.58 g/ml). Figure 4A. T_4 bacteriophage DNA. Figure 4B. Shrimp baculovirus DNA.

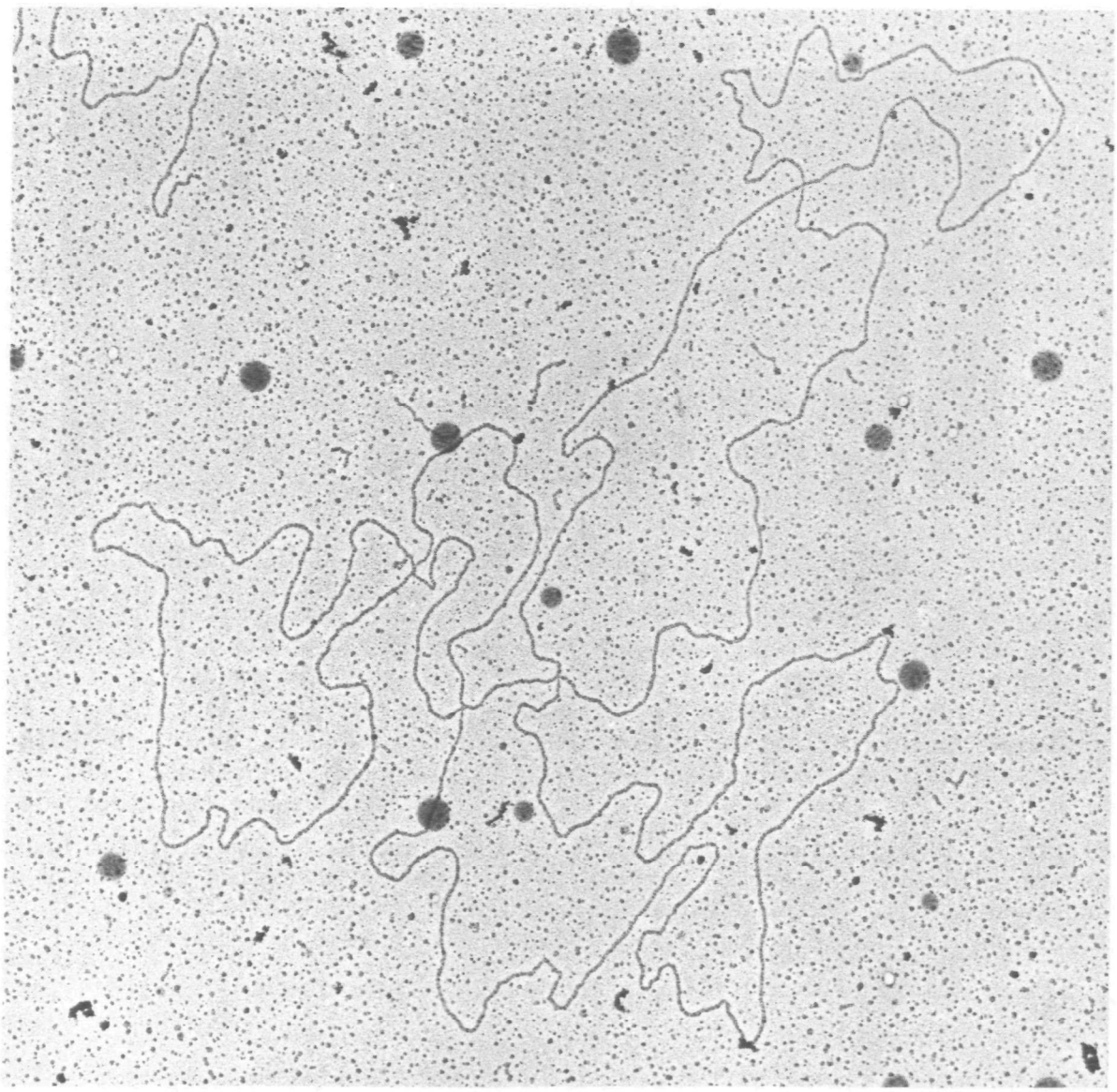

Figure 5. Kleinschmidt preparation of shrimp baculovirus DNA.
1 micron = 29.4 mm

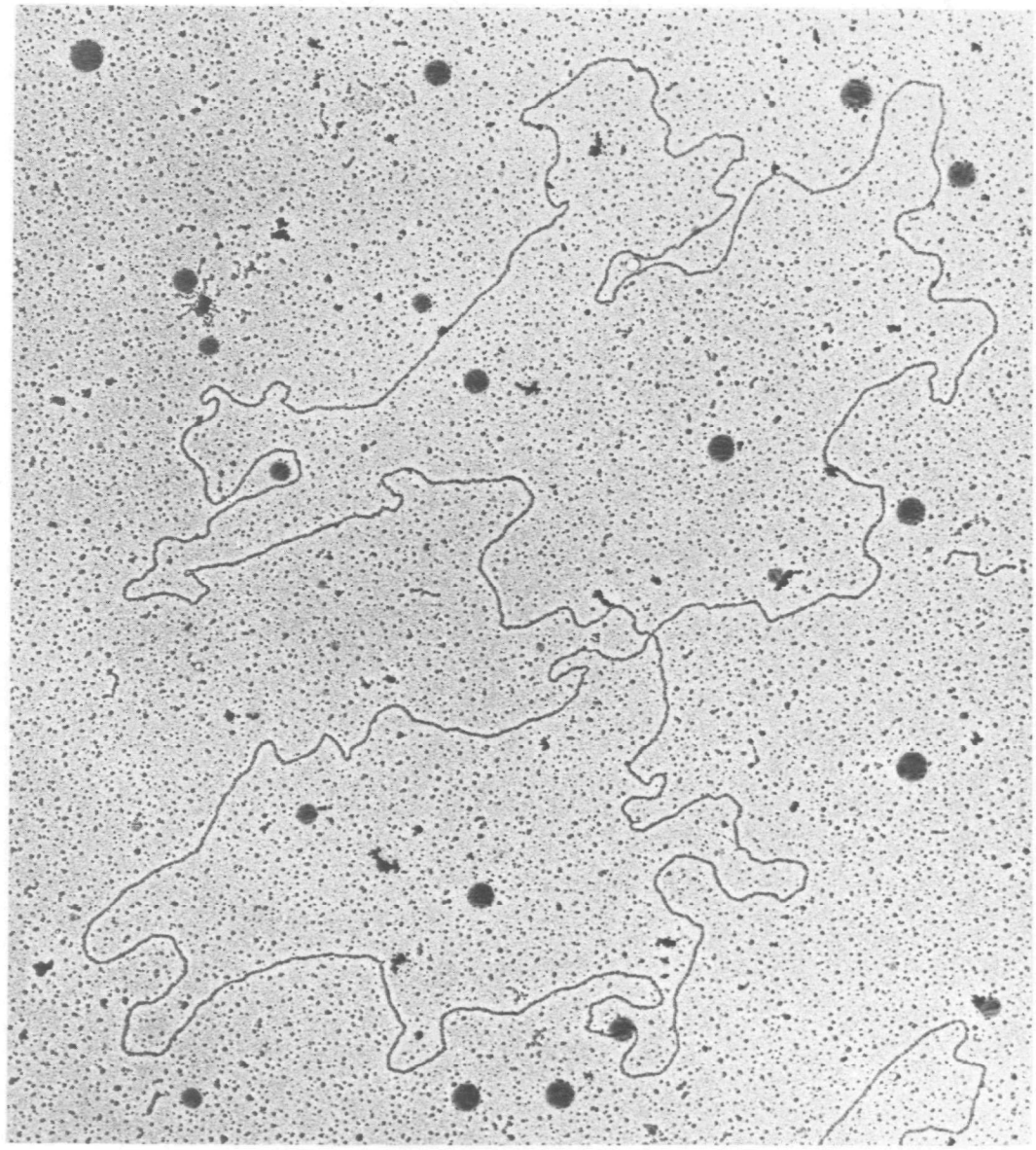

Figure 6. Kleinschmidt preparation of shrimp baculovirus DNA.
1 micron = 15.8 mm

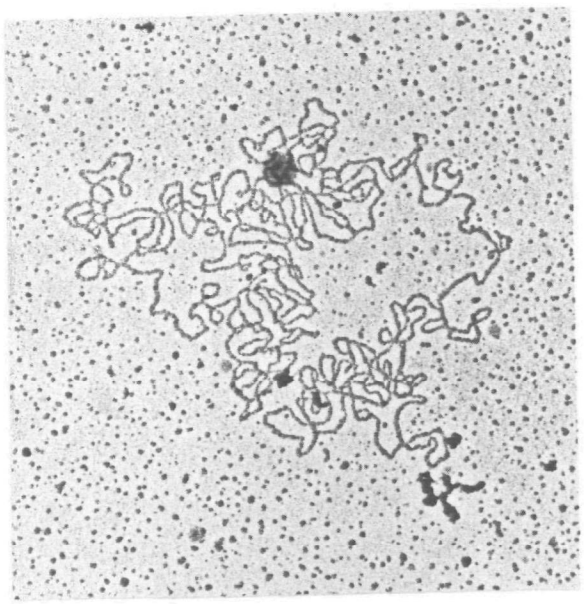

Figure 7. Kleinschmidt preparation of shrimp baculovirus DNA.
1 micron = 89.3 mm

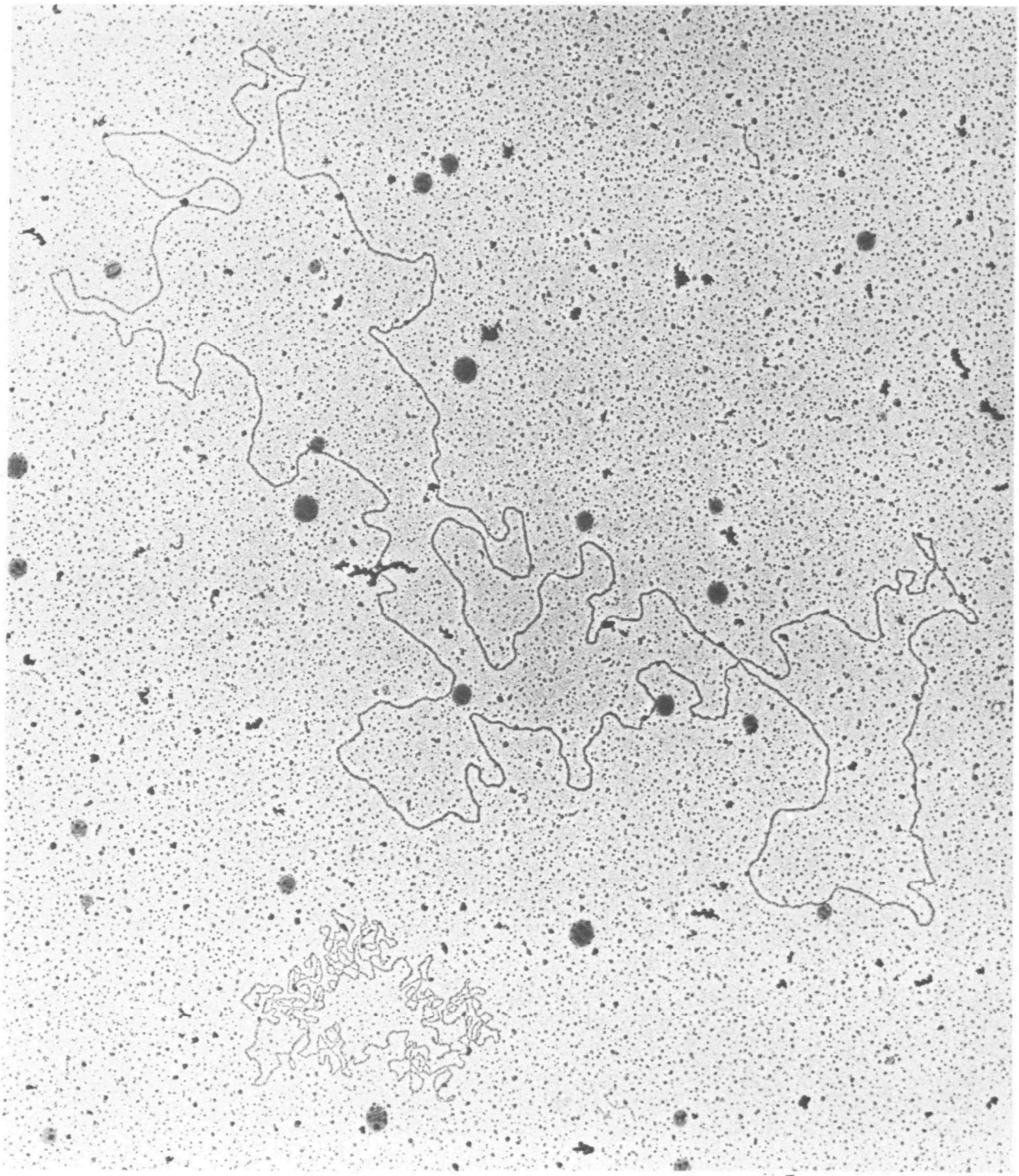

Figure 8. Kleinschmidt preparation of shrimp baculovirus DNA.
1 micron = 16.2 mm

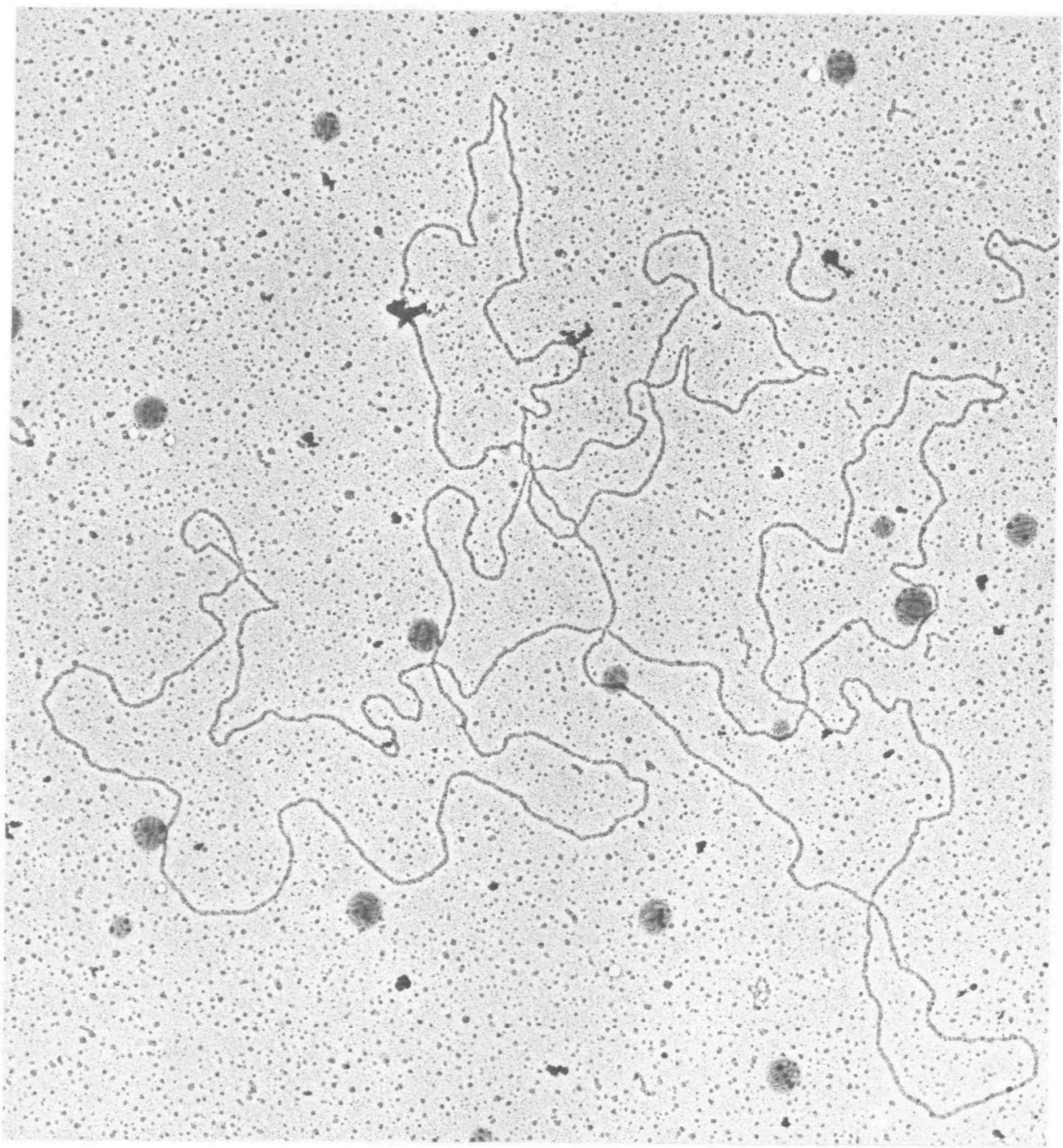

Figure 9. Kleinschmidt preparation of shrimp baculovirus DNA.
1 micron = 16.6 mm

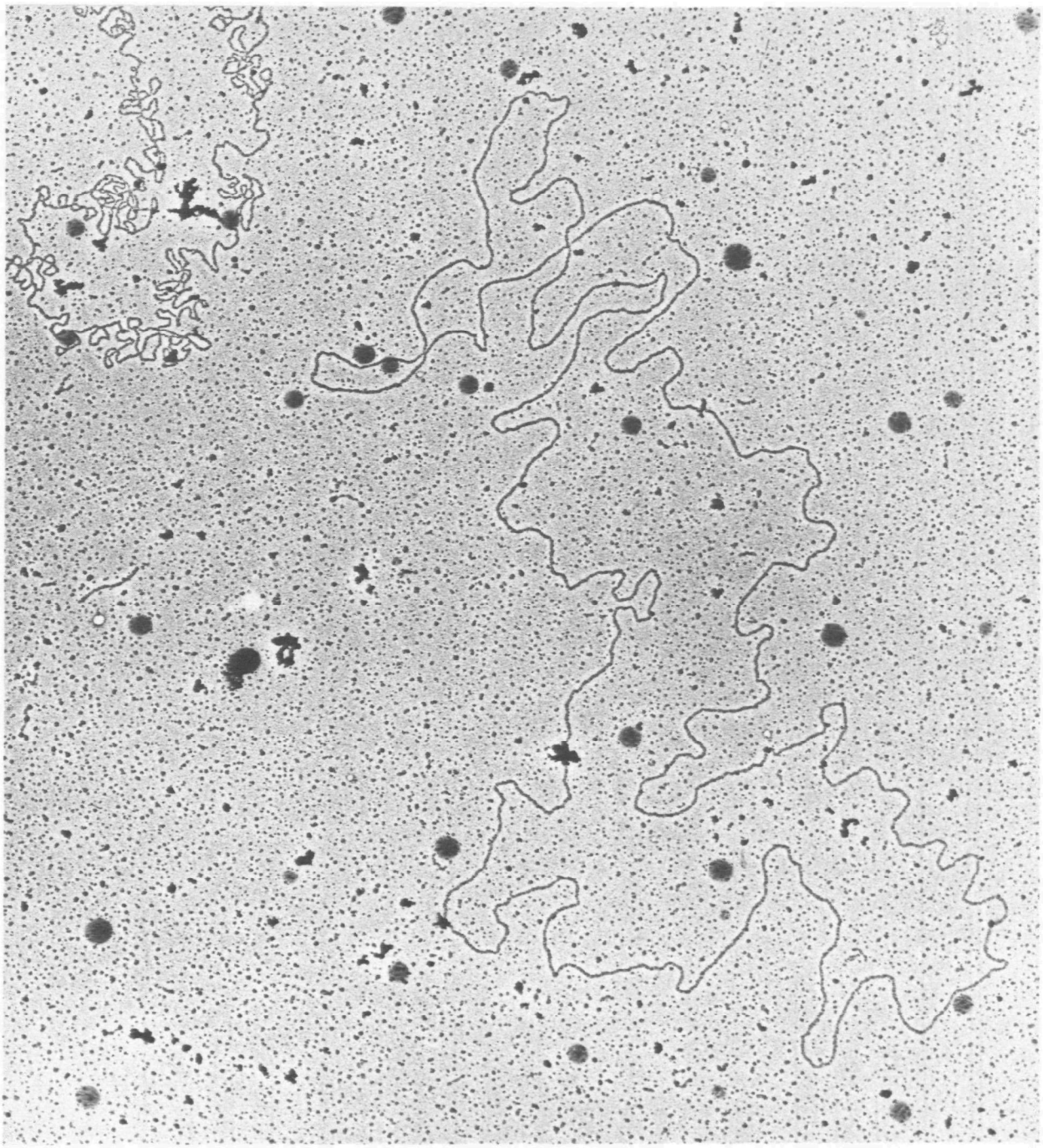

Figure 10. Kleinschmidt preparation of shrimp baculovirus DNA.
1 micron = 15.4 mm

TABLE 3. DEOXYRIBONUCLEIC ACIDS OF BACULOVIRUSES*

Virus	Size (x 10^6)	$^{S}w,20$	Reference
Agrotis segetum NPV	30	-----	(24)
Aporia crataegi NPV	58	-----	(1)
Autographa californica NPV	86-118	-----	McCarthy (personal communication)
Bombyx mori NPV	76	-----	(1)
Bombyx mori NPV	2.0	13.1S	(20)
Bombyx mori NPV	up to 10	14.5S	(5)
Bombyx mori NPV	24 and 48†	35S, 44S	(30)
Bombyx mori NPV	100†	-----	(17)
Bombyx mori NPV	59-118†	140S, 94S, 61S, 45S, 14S	(18)
Heliothis armigera	50	-----	(22)

*Table from Summers, 1977
†Presence of circular DNA molecules

(continued)

TABLE 3 (continued)

Virus	Size (x 10^6)	$^{s}_{w,20}$	Reference
Lymantria dispar MNPV	56	-----	(1)
Oryctes rhinoceros NPV	87*	57.2S	(21)
Penaeus duorarum SNPV	75*	-----	Summers and Couch (unpublished)
Rachiplusia ou MNPV	91*	58S	(27)
Spodoptera frugiperda MNPV	95*	59S	(27)
Trichoplusia ni SNPV	95*	59S	(27)
Dendrolimus sibiricus GV	80*	-----	(23)
Heliothis armigera GV	50*	-----	(22)
Spodoptera frugiperda	95*	59S	(25) (27)
Trichoplusia ni GV	100*	60S	(25) (26)

*Presence of circular DNA molecules

(continued)

TABLE 3 (continued)

Virus	Tm (host)*	G&C (%)	Density (host)*	% DNA	Reference
Aporia crataegi NPV	---------	------	-----	9	(14)
Bombyx mori NPV	86-87	41.9	-----	-----	(20)
Bombyx mori NPV	87.5	------	-----	-----	(18)
Bombyx mori NPV	---------	42.7	-----	13	(1)
Heliothis armigera NPV	71†	------	-----	-----	(22)
Hemerocampa pseudotsugata NPV	---------	------	1.710 (1.695)	-----	(10)
Lymantria dispar NPV	---------	------	-----	16.6	(1)
Oryctes rhinoceros NPV	---------	43	-----	-----	(21)
Rachiplusia ou MNPV	71.5†	43	-----	-----	(27)
Spodoptera frugiperda MNPV	72.8†	46	-----	-----	(27)
Spodoptera littoralis NPV	87.5 (83.5)	------	1.704 (1.698)	-----	(16)
Heliothis armigera GV	72†	------	-----	-----	(22)
Spodoptera frugiperda GV	74.5†	50	-----	-----	(25)
Trichoplusia ni GV	69.3†	37.5	1.6994	-----	(25) (26)

*Density of host DNA is placed in parenthesis for comparison with viral DNA.
†Determination made in 0.1 x SSC.

techniques utilizing sedimentation velocity. With this in mind, and with the molecular weight estimate derived herein, it is very likely that the shrimp baculovirus DNA falls very nicely into the size range of typical insect baculovirus DNAs.

Because of the limited amount of baculovirus DNA available for these studies, it was not possible to explore additional chemical and physical properties of the shrimp virus DNA. However, recent advances in molecular biology involving in vitro labeling of DNA combined with restriction endonuclease or physical mapping now may allow for comparisons between shrimp baculovirus DNAs and insect virus DNAs.

SEROLOGY

Immunodiffusion Assays

It has been determined in this research that PdSNPV polyhedrin is extremely insoluble to the routine DAS solubilization procedure. Consequently, initial attempts to reveal relatedness of shrimp NPV and insect NPV polyhedrins by immunodiffusion were inconclusive and in general demonstrated no cross-reaction. Therefore the use of detergent was necessary for a more complete disaggregation or solubilization of the shrimp NPV polyhedrin and experiments were conducted in the presence of various concentrations of SDS in an attempt to reveal any similar antigenic determinants in the shrimp polyhedrin. The results in Figure 11 show that after treatment with 0.1% SDS shrimp polyhedrin reacts against AcMNPV polyhedrin antisera. In Figure 11 the effect of the SDS treatment also unmasks a reaction with TnGV granulin antisera but no reaction with 1% NP-40 treated or untreated shrimp polyhedrin. A control (not shown) was conducted wherein the homologous antigen and antiserum were tested in the presence of 0.1% SDS. This did not reveal any artifacts.

These cross-reactions correlate with immunodiffusion analyses of AcMNPV and TnGV antisera. Both insect baculovirus antigens and antisera cross-react showing a strong partial identity (Summers and Hoops, unpublished data). Therefore, one would expect that related sequences present in shrimp polyhedrin would also react with the two insect baculovirus polyhedra antisera. This reaction was confirmed.

The control studies and conditions discussed in figure legends showed that 0.1% SDS had no deleterious effects on the antigen-antibody reaction, although the precipitant bands appeared slightly more diffuse than those occurring with the untreated proteins. Furthermore, preimmune sera did not react with any of the SDS-treated polyhedrins or granulins tested. Also, the 1.0% NP-40 treatment did not facilitate any reaction in the immunodiffusion assay.

Immunodiffusion assays were also performed with fractionated infected shrimp hepatopancreatic tissue. The pellets obtained by differential centrifugation of the tissue at 8,000 and 100,000 x g and the supernatant of the 100,000 x g spin were tested against AcMNPV polyhedrin, virus, and TnGV granulin antisera. All samples were again treated with 0.1% SDS prior to

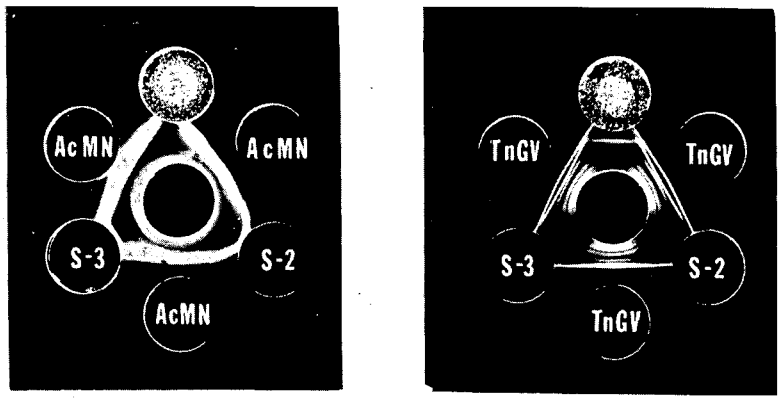

Figure 11. Immunodiffusion of shrimp polyhedrin. Figure 11A. Center well, AcMNPV polyhedrin antiserum; well 1, untreated shrimp polyhedrin; wells 1, 3, and 5, AcMNPV polyhedrin; wells 2, 4, and 6, 0.1% SDS-treated shrimp polyhedrin. Figure 11B. Center well, TnGV granulin antiserum; wells 1, 3, and 5, TnGV granulin; well 6, 0.1% SDS-treated shrimp polyhedrin; well 2, 1% NP-40 treated shrimp polyhedrin; well 4, untreated shrimp polyhedrin.

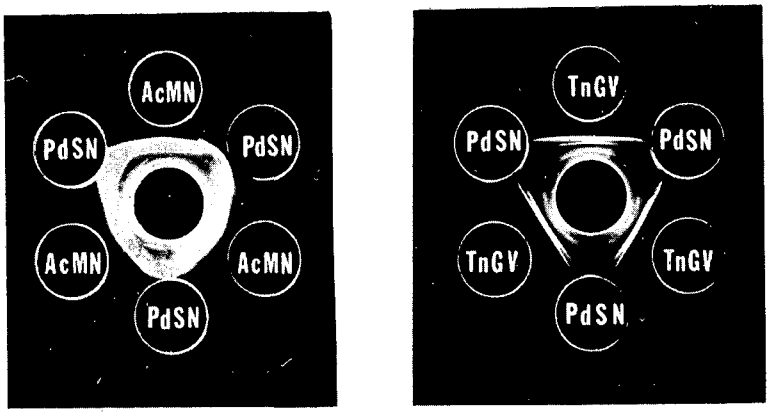

Figure 12. Immunodiffusion of infected shrimp hepatopancreatic tissue. Figure 12A. AcMNPV polyhedrin antiserum, center well; wells 2, 4, and 6, AcMNPV polyhedrin; well 1, 8000 x g infected shrimp hepatopancreatic tissue pellet; well 3, 8000 x g shrimp supernatant; well 5, 100,000 x g shrimp tissue pellet. Figure 12B. TnGV, center well; wells 2, 4, and 6, TnGV granulin; well 1, 8000 x g infected shrimp hepatopancreatic tissue pellet; well 3, 8000 x g shrimp supernatant; well 5, 100,000 x g shrimp tissue pellet.

assay. Cross reactions with both AcMNPV polyhedrin and TnGV granulin are observed in Figures 12 A-B. AcMNPV polyhedrin appears to react with all three fractions of the infected hepatopancreatic tissue whereas TnGV reacts only with the 8,000 x g pellet. The reasons for these differences are unclear at present.

The fractionation protocol for infected shrimp tissue (Figure 2) was intended primarily to concentrate nonoccluded PdSNPV virions for a comparison with AcMNPV enveloped nucleocapsid antisera. The reactions not shown here were all negative, implying two possibilities: 1) there was no serological relationship between or among viral antigenic determinants, or 2) the concentration of virus protein in the various fractionations was not adequate to reveal any cross-reactions. Because of the latter possibility, further studies are needed with greater quantities of shrimp hepatopancreatic tissue before more definitive conclusions can be made. Although not available at present, antisera are being prepared against shrimp baculovirus polyhedrin and infected hepatopancreatic tissue. Once the antisera have been collected and tested for titer, a complete comparison of the shrimp baculovirus system with insect baculovirus antigens will be rerun.

Since the insect baculovirus polyhedrins for AcMNPV and TnGV possess common primary sequences (Summers and Smith, 1976), the cross reactions with those two antisera reveal that relatedness in the primary structure between the two proteins does exist and is detected by the immunodiffusion assay. Therefore, cross reactions obtained with PdSNPV polyhedrin against both of the insect baculovirus antisera indicate that the shrimp polyhedrin contains some similar primary sequence(s) and therefore common or similar antigenic determinant(s) to those found in insect baculovirus polyhedrins and granulins. However, a careful analysis of the results also suggests that PdSNPV polyhedrin may contain different antigenic determinants. Further studies are needed to clarify these relationships.

Radioimmunoassay (RIA)

Figure 13 shows the results of competition RIA of shrimp polyhedrin versus AcMNPV polyhedrin and its homologous antiserum. The results show that if shrimp polyhedrin is not treated with SDS in order to denature the protein and expose reactive sites, it does not compete in the RIA. However, if treated with 0.1% SDS, it does compete but at a level 5,000-fold greater than the homologoud system. This confirms that some related sequence is available in the shrimp polyhedrin protein. This technique is quantitative, reproducible, and sensitive. The comparisons with other insect NPVs are utilized to reveal the degree of relatedness of the shrimp polyhedrin. As can be observed, shrimp polyhedrin is the least related of all the baculovirus polyhedrins investigated to date.

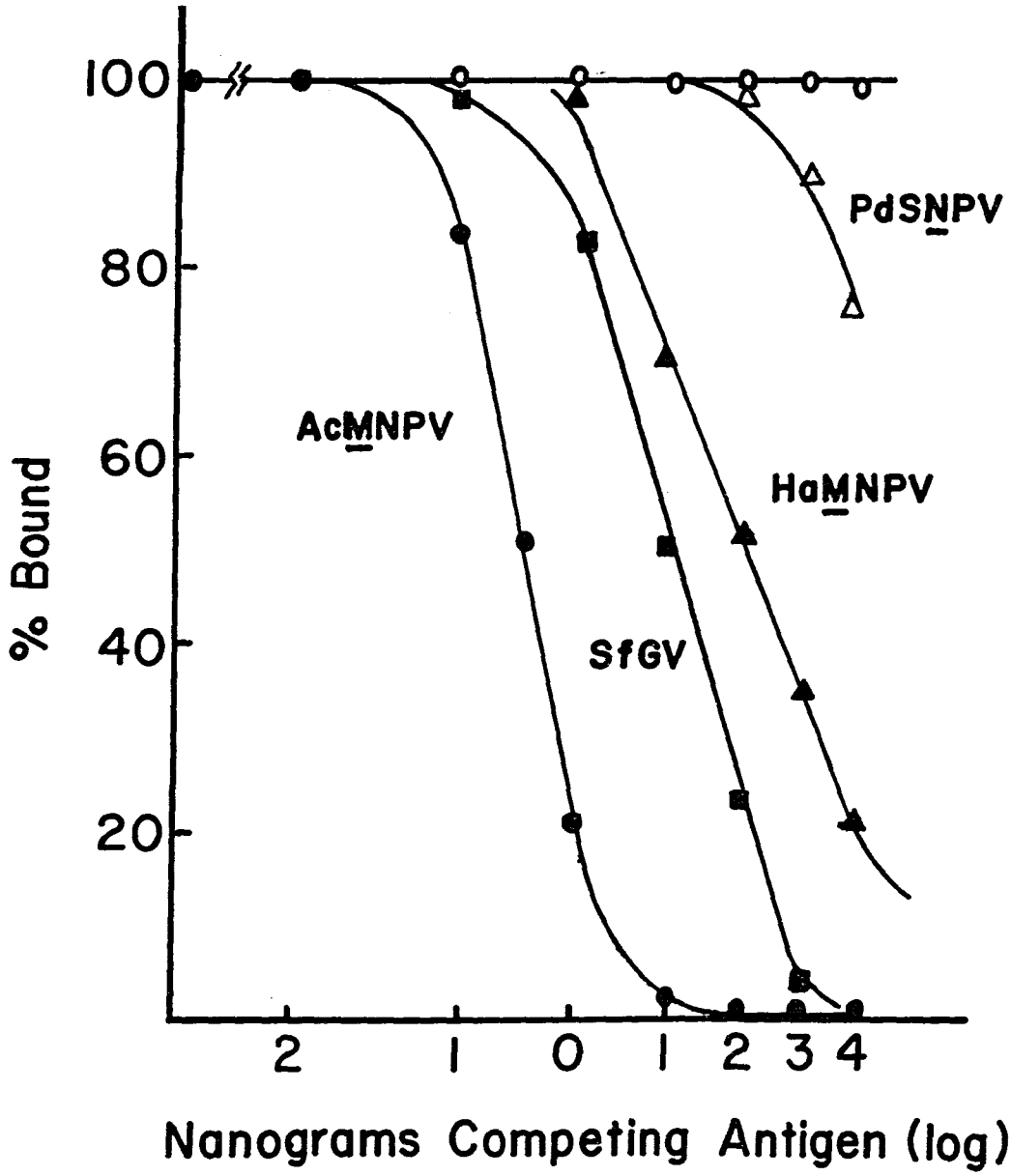

Figure 13. Analysis of shrimp polyhedrin by competition radioimmunoassay with anti-AcMNPV polyhedrin. The assay employed 1 ng of ^{125}I-AcMNPV polyhedrin (80,000 cpm/ng) and anti-AcMNPV polyhedrin serum (1:2500 final dilution). The competing proteins, purified AcMNPV polyhedrin (●), HaMNPV (▲), SfGV granulin (■), and shrimp polyhedrin (△), were added in increasing 10-fold concentrations as indicated.

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16. ABSTRACT

The research undertaken involved the partial characterization of a baculovirus of the pink shrimp, Penaeus duorarum. The significance of the study is related to the fact that the shrimp baculovirus is morphologically similar to insect vaculoviruses which were considered unique to insect arthropods prior to the discovery of shrimp nuclear polyhedrosis baculovirus (NPV). Further, insect baculoviruses are being developed and applied as microbial pesticides for the control of certain agricultural insect pests. Whereas the baculovirus diseases in pests of agricultural or medical importance are considered a desirable relationship, a baculovirus infection in shrimp is an undesirable one.

Research included investigations of the biochemical, structural, and, where appropriate, biological properties of the shrimp virus as compared to those of known and characterized properties of insect baculoviruses, both granulosis and NPVs.

Evidence for any structural relatedness of the shrimp NPV to insect NPVs has been confirmed in cross-reactions of purified shrimp NPV polyhedrin and infected shrimp tissues to insect baculovirus antisera.

This report covers the period September 23, 1974 to December 31, 1976.

17. KEY WORDS AND DOCUMENT ANALYSIS

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