

AGGLUTINATION OF VERTEBRATE ERYTHROCYTES BY THE  
GRANULOSIS VIRUS OF PLODIA INTERPUNCTELLA

by

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TABLE OF CONTENTS

	<u>Page</u>
I. PREFACE . . . . .	1
II. INTRODUCTION . . . . .	3
III. LITERATURE REVIEW . . . . .	7
IV. AGGLUTINATION OF VERTEBRATE ERYTHROCYTES BY THE GRANULOSIS VIRUS OF <u>PLODIA</u> <u>INTERPUNCTELLA</u> . . . . .	38
Abstract . . . . .	39
Introduction . . . . .	40
Methods . . . . .	42
Results. . . . .	48
Discussion . . . . .	50
Figures and Tables . . . . .	57
V. LITERATURE CITED . . . . .	70
ABSTRACT OF THESIS . . . . .	1

## SECTION I

## PREFACE

## PREFACE

The research data in this thesis is presented in the form in which it was submitted for publication. This work has been submitted to and accepted for publication in the journal, Virology.

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SECTION II  
INTRODUCTION

## INTRODUCTION

Viruses that characteristically infect and replicate in invertebrate hosts represent a unique class of animal viruses. Currently, 650 different insect viruses, classified into eight families, are recognized: 1) Reoviridae; 2) Rhabdoviridae; 3) Togaviridae; 4) Parvoviridae; 5) Picornaviridae; 6) Poxviridae; 7) Iridoviridae; 8) Baculoviridae. Of these, the baculoviruses have received the most attention because of their potential for use as biological insecticides (Falcon, 1976). The baculoviruses are unusual animal viruses because the virions are embedded in a proteinaceous matrix that is believed to stabilize the virus in environments outside the insect host. There are three subgroups within the baculovirus family that are distinguished by the rod-shaped nucleocapsid structure: 1) nuclear polyhedrosis viruses (NPV); 2) granulosis viruses (GV); 3) Oryctes and braconid viruses. The primary difference between these viruses lies in the number of virions occluded per matrix. Granulosis virus enveloped nucleocapsids are singly occluded, whereas, in NPVs, many virions are embedded either as groups or randomly within the matrix (Harrap and Payne, 1979; Maramarosch, 1968; Summers, 1977). The Oryctes virus is unusual in that the enveloped nucleocapsids are not occluded in a protein matrix; they are classified with the baculoviruses because of the rod-shaped capsid structure (Payne et al., 1977).

The host specificity of insect viruses ranges from very diverse to very specific. The entomopoxviruses, iridoviruses and NPVs exhibit the widest divergence of host specificity (David, 1975; Smith, 1976), whereas the granulosis viruses appear to be restricted solely to specific lepidopteran insects (David, 1975). In addition, baculoviruses as a group are apparently restricted to invertebrate hosts (Stoltz and Vinson, 1979; Wildy, 1971). There are no reports of vertebrate viruses with similar morphology and characteristics. The limited host range of these viruses with respect to invertebrate and vertebrate hosts makes the baculoviruses, particularly the granulosis viruses, attractive candidates for biological insecticides (FAO/WHO, 1973).

Interest in development of insect viruses as biological control agents has encouraged investigation of the molecular biology of GVs. Such studies are necessary to properly assess the impact of these viruses on non-target hosts and on the environment as well as determining their effectiveness in controlling insect pests.

Fundamental research on the biochemical and biophysical properties of baculoviruses has concentrated mainly on the NPVs because several host cell systems are available for in vitro propagation of these viruses. Unfortunately, investigations of granulosis viruses at the cellular and molecular level have been limited because GVs as a group do not appear to infect cells in vitro, and no suitable tissue culture system has yet been found to support their replication. The evaluation of factors that may influence viral infection and replication

is necessary if propagation of GVs in vitro is to be successful. Because one of the first steps in the infection process is adsorption of the virion to the cell, the problem of in vitro infection with GVs could reside in the initial interactions of the virus with the cell, i.e., conditions and mechanism of adsorption to the host cell. In several instances, viral adsorption has been mediated through the same receptors responsible for hemagglutination (Howe and Lee, 1970). Thus, the ability of Plodia interpunctella GV to agglutinate vertebrate erythrocytes was studied in an attempt to understand the mechanisms of and conditions required for viral adsorption. Such knowledge is not only vital to establishment of an in vitro system, but also is necessary in accessing potential hazards associated with infection of non-target hosts.

SECTION III  
LITERATURE REVIEW

## LITERATURE REVIEW

Granulosis Viruses as Biological Insecticides.

The pathology of granulosis virus infection was first observed in larvae of the large white butterfly, Pieris brassicae by Paillot in 1926. Some years later a similar disease was found in a cutworm, Agrotis segetum (Paillot, 1934), but was not confirmed until Steinhaus (1947) described the same pathology in the variegated cutworm, Peridroma margaritosa. The disease was given the name granulosis because infected tissue sections observed under light microscopy revealed cells packed with minute, refractile bodies or "granules". The viral character of these granules was demonstrated by Bergold (1948), who examined infected tissue of the pine shoot roller, Choristoneura marinana, by electron microscopy and found rod-shaped virus particles present in the tissue.

At the present time, over 80 species of lepidopteran insects have been found susceptible to granulosis viruses (Martignoni and Iwai, 1977). Many of these species are economically important pests that are highly susceptible to granulosis infection. The use of viruses in controlling insect populations has gained favorable attention recently due to the rapid development of insects resistant to conventional insecticides, i.e., chemical agents such as synergized pyrethrins or malathion, the only insecticide widely accepted for protection of stored commodities (FAO/WHO, 1973). Furthermore,

mounting concern regarding hazardous effects of chemical insecticides on the environment has encouraged development of integrated pest control programs which incorporate biological agents.

Insect viruses of the family, Baculoviridae, are likely candidates for viral insecticides for several reasons. In vivo and in vitro studies have demonstrated the effectiveness of GV in eliminating the target hosts. The GV of P. brassicae and P. rapae have been demonstrated to be effective against their specific hosts (Biliotti et al., 1956; Kelsey, 1957; 1958; Wilson, 1960). In field studies, GVs of the cotton cutworm, cereal noctuid and the cabbage looper have been used to control infestations and limit crop destruction (FAO/WHO, 1973; Ito, 1977; Ito et al., 1977). Large scale programs utilizing GVs have been successfully employed in the Soviet Union, Czechoslovakia, Yugoslavia, People's Republic of China, Australia, and Great Britain (Ito et al., 1975). For example, the GV of the fir budworm, C. muriana and the potato moth GV has been utilized in Canada and Australia, respectively. However, such programs have been limited in Western Europe and the United States due to more stringent registration requirements (EPA, 1975; Franz, 1976; Ignoffo, 1973; Krieg, 1976; Rivers, 1976).

Of the baculoviruses isolated thus far, only the nuclear polyhedrosis viruses of Heliothis zea, Hymantia dispar and Orgyria pseudosugata have been approved and utilized in biological control in the United States due to more stringent registration requirements (EPA, 1975; Shieh and Bohmfalk, 1980). At the present time, the GV of the

codling moth, Laspeyresia pomonella, has been used in field studies and is nearing commercial application (Brassel, 1978; Falcon, et al., 1968; Huber and Dickler, 1975; 1977; Jaques et al., 1977; Kurstak, 1970). The GV of the Indian meal moth, Plodia interpunctella, has been studied for its insecticidal potential. The effectiveness of P. interpunctella GV has been demonstrated in corn and wheat (Kinsinger and McGaughey, 1976; McGaughey, 1975a), and in almonds and raisins (Hunter et al., 1973; 1977; 1979).

The insecticidal potential of the granulosis viruses is just now being realized. As research progresses, there are prevailing considerations regarding overall safety and effectiveness of these agents in integrated pest management programs. Unlike many vertebrate viruses, very little is known of the cellular and molecular biology of the baculoviruses. In particular, there is little or no information concerning detrimental effects on non-target hosts at the cellular and molecular level. Previous studies have attempted to access possible effects of these viruses on other organisms (Banowetz et al., 1976; Burges and Hussye, 1971; Huber and Krieg, 1978; Ignoffo, 1972; 1973; Krieg, 1976; Lautenschlager et al., 1977; Röder and Pünter, 1977). There is some evidence suggesting incomplete NPV replication in a vertebrate viper cell line (McIntosh and Maramorosch, 1973) and in CHO cells (McIntosh and Shamy, 1980), and of persistence of viable NPV in cell cultures of human lung, leukocyte and amnion tissues (McIntosh and Shamy, 1975). However, it must be emphasized that such



tests have not properly evaluated the effects at the cellular and molecular level. The possibilities of recombination involving viral and host genomes, latency and integration, and toxicity of various viral components have not been investigated.

Under present guidelines as proposed by the Environmental Protection Agency, the safety of disseminating GV's into the environment with consequent exposure of man, animals and plants to exogenous agent can not be fully investigated until each virus can be identified (FAO/WHO, 1973). Unequivocal identification of these viruses as well as accessing possible interactions with non-target species both in vivo and in vitro is dependent upon prior characterization of the viral nucleic acid, structural and non-structural proteins and other components. Characterization of GV's is also necessary to: 1) enable detection of mutants which may arise during development and production as insecticides which possess increase virulence or alterations in host range; 2) allow development of assays for detecting viral residues; 3) permit monitoring of insect resistance to viral strains.

Basic research on the NPV's and GV's has been fostered by their potential for use as virocidal agents, particularly against chemically-resistant insects. Pests of stored grain represent one such group of insects which are becoming difficult to control by chemical means. A stored grain insect which can not be controlled by approved chemicals such as malathion, and is economically important to grain-producing states is the Indian meal moth, P. interpunctella (Zettler et al., 1973).

Thus, it is of great interest to develop alternative means of controlling this pest without damaging the stored product. Arnott and Smith (1968a) described a granulosis virus of P. interpunctella that appears to satisfy the criteria of an effective biological insecticide. The virus has been shown to be highly virulent and specific for P. interpunctella, and in laboratory studies effectively controlled insect populations in stored nuts (Hunter et al., 1973), and surface layers of corn and wheat (McGaughey, 1975). Kinsinger and McGaughey (1976) have demonstrated that the P. interpunctella GV will retain its biological activity under normal field conditions, and that effective doses of the virus are low and are compatible with several chemical agents. These studies suggest that P. interpunctella GV is effective as a biological control agent implemented in the field; however, knowledge of the physical and biological characteristics of P. interpunctella GV was, until recently, severely lacking. Although studies of the in vivo infection process of P. interpunctella GV have been reported (Arnott and Smith, 1968a; 1968b; Hunter and Drexel, 1972), its effect on non-target hosts (invertebrate, vertebrate and plants) has not been investigated. Such studies are necessary before this virus can be utilized safely and effectively in the field.

## Cellular and Molecular Biology.

### I. Structure and Composition.

#### Occluded Virus.

The structure of granulosis viruses has been examined extensively using electron microscopy (Bergold, 1948). GVs are ovocylindrical in shape and are 300 to 500 nm in length by 120 to 350 nm in width (Bergold, 1963; Huger, 1963; Tweeten et al., 1977a). Electron microscopy of thin sections of isolated virus or of infected tissue revealed GV to be structurally complex (Arnott and Smith, 1968a; 1968b; Asayama, 1976; Bergold, 1963; Morgan et al., 1955; Stairs et al., 1966; Summers and Paschke, 1970; Tweeten et al., 1977a). These viruses consist of rod-shaped nucleocapsids (N) surrounded by a unit membrane envelope. Each enveloped nucleocapsid (EN) is embedded within a matrix of protein having a regular crystalline lattice. Interestingly, the continuity of the lattice pattern is not disrupted by the enveloped nucleocapsids (Bergold, 1963b). This protein matrix is unique to certain invertebrate viruses such as the baculoviruses and is believed to contribute to their overall stability in the environment. As seen by electron microscopy, there is a distinct electron dense layer at the periphery of the protein matrix (Arnott and Smith, 1968a; Kawanishi, et al., 1972a), which may be condensed matrix protein (Harrap, 1972a), or a lipoprotein membrane (Hess and Falcon, 1978). The entire particle has been generally referred to as the occluded envelope nucleocapsid

although "inclusion body" or "capsule" have been used to describe the entire virus.

Characterization of such features as structure, protein composition and nucleic acid is dependent upon the isolation and purification of the virus from its host. With most viruses this involves separating the virus from infected cells grown in vitro. Unfortunately, GVs as a group have not been found to replicate in vitro so that investigators to date have necessarily used virus from infected larvae. Several methods have been developed for purification of occluded virus from larvae (Harrap, 1972a; Harrap and Longworth, 1974; Khosaka et al., 1971; Summers and Egawa, 1973; Tweeten et al., 1977a). These procedures generally include homogenization of larvae followed by several steps of differential centrifugation and/or velocity sedimentation in gradients. However, few of these methods have been critically examined for efficiency and degree of purification. Tweeten et al., (1977a) demonstrated the adequacy of one such procedure developed for the GV of P. interpunctella. Mixing experiments employing uninfected, radioactively labeled larvae and infected, unlabeled, larvae demonstrated the effectiveness of the protocol in removing contaminating host protein. The use of detergent (1% deoxycholate, vol/vol) was found to be indispensable in isolating virus free of host material. Furthermore, the virus retained its physical and biological integrity.

Dissociation of the purified, occluded virus into envelope nucleocapsids and protein matrix in vitro is accomplished by exposure to sodium carbonate buffers, pH 9 to 11 (Bergold, 1947; Egawa and Summers,

1972; Harrap, 1972b; Khosaka et al., 1971; McCarthy and Liu, 1976; Summers and Paschke, 1970; Tweeten et al., 1978). It appears that dissolution of the protein matrix by carbonated buffers closely mimics the solubilization and release of envelope nucleocapsids as observed in vivo. Other chemicals such as 5 M guanidine, 7 M urea, and n-propanol have been employed; however, damage to the virus has been reported using such agents (Egawa and Summers, 1972; Kawanishi, et al., 1972a). Recently, Yamamoto and Tanada (1978a) have shown that the matrix of Pseudaletia unipuncta GV is solubilized by 0.02 M NaOH, pH 12, with no detrimental effect on the viral envelope.

Optimal conditons for dissolution of the matrix must be determined for each GV, for the rate of solubilization is dependent on temperature, pH and carbonate concentrations (Egawa and Summers, 1972). Tweeten et al. (1978), found that 0.05 M NaCO<sub>3</sub>, 0.05 M NaCl, pH 10.6, permitted optimal solubilization of P. interpunctella GV protein matrix over a 5 to 15 minute period at 20 to 22°. Once the matrix has been solubilized, the envelope nucleocapsids may be separated from the matrix proteins by centrifugation either by velocity sedimentation on sucrose gradients or by differential centrifugation. The matrix proteins are recovered from the top of the gradients or in the supernatant while the EN can be found in the gradient or in the pellet.

#### Protein Matrix Proteins.

The protein matrix surrounding the envelope nucleocapsid is

the most predominant and best characterized protein in GV's. Alkali solubilization of the matrix appears to generate a heterogeneous mixture of polypeptides which have been examined by gel electrophoresis and sedimentation analysis (Longworth et al., 1972; Summers and Egawa, 1973; Tanada and Watanabe, 1971). Carboxy- and amino-terminal analysis of solubilized matrix indicated multiple peptide species, and serological assays such as agar-gel immunodiffusion demonstrated that at least two antigenic species are generated.

The discovery of alkaline proteases associated with the protein matrices which digest the matrix during solubilization has permitted further biochemical characterization of the matrix (Eppstein and Thoma, 1975; Summers and Smith, 1975a; 1975b). Several baculoviruses have been found to have these proteases (Crawford and Kalmakoff, 1977; Eppstein et al., 1975; Kozlov et al., 1975; McCarthy and Liu, 1976; Tweeten et al., 1978). These proteases have been shown to be activated under alkaline conditions required to solubilize the matrix and appear to cleave the matrix into lower molecular weight polypeptides. Use of protease inhibitors such as HgCl or heat treatment (70° for 30 min) has permitted solubilization and recovery of the matrix protein in the nondegraded form (Eppstein et al., 1975; Summers and Smith, 1975b; Tweeten et al., 1978). The nondegraded matrix is composed of a single 12S component which is further dissociated by sodium dodecyl sulfate (SDS) and 2-mercaptoethanol into one low molecular weight polypeptide (Summers and Smith, 1975a; 1975b). The

matrix proteins of several granulosis viruses have been examined, and the molecular weight of the nondegraded polypeptide or granulin fall within the range of 25,000 to 30,000 daltons (Brown et al., 1977; Croizier and Croizier, 1977; Summers and Smith, 1975a; 1975b; 1978; Tweeten et al., 1978, Yamamoto and Tanada, 1978c). These results suggest that the 12S molecule liberated from non-degraded protein matrix may consist of eight granulin subunits. Similar observations have been made regarding the polyhedrin molecule of the NPVs. Furthermore, immunochemical studies with the polyhedrins of two Orgyia pseudotsugata NPVs and Trichoplusia ni NPV indicate that the 12S molecule contains at least one of the major antigens observed in solubilized matrix preparations (Eppstein and Thoma, 1977; Rohrmann, 1977).

Analysis of peptide and amino acid composition suggests the granulins from several GV's are very similar. These proteins are high in aspartic and glutamic acid, valine, isoleucine and leucine. Comparative peptide mapping of both granulins and polyhedrins reveal many common peptides (Maruniak and Summers, 1978; Summers and Smith, 1975a). Several investigators have suggested that these common domains are evolutionarily conserved and represent regions of the protein responsible for its aggregative properties. The remaining peptides appear to be unique to each viral isolate. Summers and Smith (1975a), and Tweeten et al., (1980c), have demonstrated that the granulin of T. ni GV and P. interpunctella GV are modified by

phosphorylations. Whether this is the case with other baculovirus matrices has not been established. Indeed, the examination of granulins for other protein modifications (acetylations, methylations, etc.) has not been performed.

#### Enveloped nucleocapsids.

Examination of purified envelope nucleocapsids has revealed further structural details of the granulosis viruses. The envelope (previously referred to as the "outer" or "developmental membrane") of the GVs of *P. rapae* and *P. operculella* have been found to be triple-layered membranes 50 to 60 Å<sup>0</sup> thick which bear a close structural resemblance to biological lipoprotein unit membranes (Beaton and Filshie, 1976). The surface of these membranes do not appear to be modified by glycoprotein spikes that are commonly found on other enveloped animal viruses.

The nucleocapsids consist of a DNA core surrounded by a capsid structure composed of protein. The average dimensions of GV nucleocapsids are 30 to 60 nm by 260 to 360 nm. The rod-shaped capsid, previously referred to as the "intimate" or "internal membrane" is composed of protein subunits assembled in a regular lattice (Smith and Hills, 1962; Summers and Paschke, 1970). Diffraction studies (Beaton and Filshie, 1976), and electron microscopic measurements (Harrap and Juniper, 1966), indicate the lattice is composed of rings of subunits stacked upon one another. The nucleocapsid appears to



to have morphologically distinct ends (Kozlov and Alexeenko, 1967; Summers and Paschke, 1970). One end is blunt (the "tail-plate" or "claw") and the other consists of a capped structure (Teakle, 1969). The importance of this morphological difference has not been determined.

Tweeten et al. (1980a), have demonstrated the presence of basic proteins similar to that of protamines associated directly with the large, double-stranded DNAs of NPVs and GVs. These investigators have revealed the possible existence of a nucleoprotein complex contained within baculovirus capsids.

Procedures for isolation of envelope nucleocapsids and nucleocapsids from occluded virus have been established by several investigators. The method used can greatly influence various characteristics of the virus. Structural integrity, infectivity, and purity of EN will vary substantially depending on how the virus is manipulated (Harrap, 1972b; Kawanishi and Paschke, 1970; Kawase et al., 1973). Several investigators have found that immediate separation of the EN from the carbonate buffer (or other dissolution medium) by centrifugation yields stable virus in large quantities (Bell and Orlob, 1977; McCarthy and Liu, 1976; Summers and Paschke, 1970; Tweeten et al., 1980c). In addition, optimal pH conditions should be established to prevent: 1) precipitation of matrix protein which would contaminate the EN; 2) loss of EN due to aggregation (Arif and Brown, 1975; Harrap and Longworth, 1974; Summers and Paschke, 1970). Exposure to alkaline conditions should be brief

and dialysis avoided so as to minimize viral degradation and loss of infectivity (Tweeten et al., 1980c; Yamamoto and Tanada, 1978a). The overall stability of isolated EN varies with the viral species but is much less than that of the occluded form.

The isolation of nucleocapsids from EN requires removal of the viral envelope. The most effective agents for solubilization of membranes are detergents such as sodium dodecyl sulfate (SDS), Triton, deoxycholate or Nonidet P-40 (NP-40). Of the detergents tested, NP-40 appears best suited for efficient removal of baculovirus envelopes. In the isolation of nucleocapsids from Melcanchma persicaria GV, EN were incubated in 0.2% (vol/vol) NP-40 for 30 min prior to centrifugation (Harrap and Longworth, 1974). However, Arif and Brown (1975) found that similar conditions did not completely remove the envelope of the NPV of Choristoneura fumiferana, and the nucleocapsids were substantially degraded if higher concentrations of NP-40 were used. Modifications of the above procedures were employed in the isolation of nucleocapsids from the GVs of P. brassicae and P. interpunctella (Brown and Kelly, 1977; Tweeten et al., 1980c). Briefly, EN were incubated in 1% (vol/vol) NP-40 in 0.01 M Tris-HCl, pH 8.5 for 30 min at 22 - 30°. Velocity sedimentation in sucrose or glycerol gradients was used to separate nucleocapsids from the envelopes. The nucleocapsids were shown by electron microscopy to be structurally intact and lacking contaminating envelope fragments. Nucleocapsid aggregation was minimized by Tweeten et al. (1980c), by

maintaining the pH at 8.5 during detergent incubation and velocity sedimentation centrifugation. At pHs below pH 8.5, nucleocapsids are lost due to aggregation or clumping and will pellet during centrifugation. It must be stressed that efficient conditions for envelope solubilization varies with the species of GV involved. Yamamoto and Tanada (1979), have used 0.1% Triton X-100 in place of NP-40 to solubilize viral envelopes of P. unipuncta GVs. The GV of Spodoptera frugiperda is especially resistant to most solubilization agents used including 2% NP-40 or 6 M urea (Summers and Smith, 1978).

Capsids from GVs have been isolated using treatments combining salt shock and detergent (Summers and Smith, 1978). Envelope nucleocapsids are incubated in 2% NP-40 and 1.0 M NaCl for 18 hours at 37° after which the preparation was applied to preformed cesium chloride gradients. Summers and Smith (1978), reported that the tubular capsids banded at 1.33 g/ml. Tweeten et al. (1980a), produced capsids from P. interpunctella GV from nucleocapsids using essentially the treatment of Summers and Smith (1978), except that the detergent was omitted. The conditions used were sufficient to release the DNA and any core proteins from the capsids. Under electron microscopy, the capsids appear as empty, tubular structures which have lost the capped structure located at the ends of nucleocapsids. Whereas the capsid structure is stable under high salt conditions, these capped structures are not.

### Envelope Proteins.

Proteins specific to envelope nucleocapsids have been characterized for only a few GV's. The enveloped nucleocapsids of S. frugiperda, T. ni, P. brassicae, P. unipuncta and P. interpunctella GV's have been purified and analyzed by polyacrylamide gel electrophoresis (PAGE) (Brown et al., 1977; Summers and Smith, 1978; Tweeten et al., 1980c; Yamamoto and Tanada, 1979). Many structural polypeptides have been identified, with each virus having a unique protein composition. Polypeptides ranged in molecular weight from 12,000 to 160,000 daltons as determined by SDS-PAGE. The GV of S. frugiperda and T. ni appear to have the greatest number of identifiable polypeptides; however, Tweeten et al. (1980c), have resolved and identified several additional minor proteins of similar electrophoretic mobility in the EN of P. interpunctella GV using SDS-polyacrylamide gradient gel electrophoresis. Their data suggest that PAGE systems capable of resolving proteins over broad molecular weight ranges should be employed in characterizing these complex viruses.

Analysis of proteins specifically associated with the viral envelope requires analysis of enveloped nucleocapsids, envelopes extracted by detergents (NP-40, Triton X-100, etc.) and nucleocapsids. SDS-PAGE analysis of these various components has revealed that four to five proteins are removed with NP-40 treatment of P. brassicae and P. interpunctella GV (Brown et al., 1977; Tweeten et al., 1980a; Tweeten et al., 1980c). Yamamoto and Tanada (1979), have reported

that P. unipuncta GV enveloped nucleocapsids, when treated with Triton X-100 detergent, yielded nine proteins associated with the envelope and only three proteins present in isolated nucleocapsids. Such discrepancies may be due to complexities or organizational differences in GV nucleocapsids, or differences in extractability of the proteins by different detergents. To rule out such problems, Tweenen et al. (1980c), employed iodination ( $^{125}\text{I}$ ) of surface envelope proteins by solid-phase lactoperoxidase-galactose oxidase system (Enzymobead<sup>R</sup>). Autoradiograms obtained from electropherograms of iodinated envelope nucleocapsids indicated five proteins were accessible on the surface of the virion. These same polypeptides, VP17, VP39, VP42, VP48 and VP97, also were the major species lost from envelope nucleocapsids treated with detergent.

There are several polypeptides which are not present in purified P. interpunctella nucleocapsids, but also were not surface iodinated with solid-phase lactoperoxidase (Tweenen et al., 1980c). These data suggest these proteins (VP16, VP38, and VP88) are associated with the envelope but are not accessible at the outer surface of the envelope, ie., internal to the envelope nucleocapsid structure. Such proteins may be part of the so-called "intermediate layer" thought to exist between baculovirus envelopes and nucleocapsids (Kawamota et al., 1977a).

Lipid content analysis of the envelopes has been restricted to a few granulosis viruses. In particular, the envelope of P. unipuncta

GV was found to contain phosphatidyl choline, phosphatidyl ethanolamine and a third phospholipid as yet unidentified (Yamamoto and Tanada, 1978a). Neutral lipid and glycolipid analysis has not been reported in the literature.

#### Nucleocapsid and Capsid Proteins.

The structural polypeptides of purified nucleocapsids from the GV of P. brassicae, P. interpunctella, and P. unipuncta have been analyzed by SDS-PAGE (Brown et al., 1977; Tweeten et al., 1980c; Yamamoto and Tanada, 1979). From three to eight polypeptides have been identified ranging from 12,000 to 73,000 daltons in molecular weight. A comparison of the electropherograms of these viruses reveals several discrepancies in some of the minor protein species. On the other hand, there is a high degree of similarity with respect to two major polypeptides, a protein of 31,000 to 34,000 daltons and a second ranging from 12,000 to 14,000 daltons. Analysis of isolated capsid structures indicate that the 31-34 K component is the major capsid protein in both T. ni and P. interpunctella GV (Summers and Smith, 1978; Tweeten et al., 1980c). A protein species of approximately the same molecular weight in isolated enveloped nucleocapsids and nucleocapsids has been identified in many NPVs and other GVs (Brown et al., 1977; Merdan et al., 1977; Payne et al., 1977), suggesting that these polypeptides are major elements of baculovirus capsids.

The other predominant protein is of low molecular weight (12,000 to 16,000) and has been observed in many baculoviruses (Brown et al., 1977; Cibulsky et al., 1977; Gaelp et al., 1977; Harrap et al., 1977; Merdan et al., 1977; Payne et al., 1977; Tweeten et al., 1980a; 1980c). The isolation and characterization of this polypeptide was first reported by Tweeten et al. (1980a). In P. interpunctella GV, the protein comprises nearly 50% of the nucleocapsid structure and migrates in SDS-PAGE as a 12,000 dalton species. The protein is not found in purified capsids which are devoid of DNA and can be extracted by treating nucleocapsids with sulfuric acid or by salt-shock treatment as used in isolating capsids. In acid-urea polyacrylamide gel electrophoresis, the protein possesses a high degree of mobility, intermediate to that of histones and protamines, with an isoelectric point of pH 9.8 to 10.0. Amino acid analysis of acid-extracted protein indicated its basic character and similarity to protamines. The data indicate this protein is a basic, arginine-rich polypeptide which is an internal or core protein associated directly with the viral DNA much like that of histones or protamines. When P. interpunctella GV nucleocapsids were gently treated with chelating agents such as EDTA or EGTA, a rupture at the ends of the capsids was observed by electron microscopy. A thick fiber of electron-dense material was observed emerging from the ruptured end. This fiber was found to be sensitive to salt which caused decondensation of the structure into what appeared to be long, thin strands of DNA. These observations suggested the

basic protein was associated with the viral DNA forming a nucleoprotein complex within the capsid structure.

Tweeten et al. (1980c) also demonstrated that proteins of similar basicity and molecular weight exist in Autographa californica NPV, P. rapae GV, and S. frugiperda NPV. Although a similar protein has not been observed in the GVs of T. ni and S. frugiperda (Summers and Smith, 1978), it appears that these basic polypeptides may be common to the baculoviruses as a group. The basic proteins may be of diagnostic value since development of the baculoviruses as biological insecticides will require an accurate means of detecting and identifying these viruses in the environment. Further analysis of the peptide structure and composition of these proteins as a group will reveal the functional nature and role in baculovirus molecular biology.

#### Nucleic Acid of Granulosis Viruses.

The nucleic acid composition of granulosis viruses was first reported by Wyatt (1952a; 1952b), who analyzed the GVs of C. muriana and C. fumiferana and found them to contain the deoxyribonucleotides A, G, C, and T. The double-stranded nature of the molecule has been demonstrated in the GV of the Siberian silkworm, Dendrolimus sibiricus and A. segetum (Shvedchikova and Tarasevich, 1968). Electron microscopic analysis indicated that these molecules were of high molecular weight. Evidence for the circular conformation of the genome of several GVs has been obtained through electron microscopy and sedimentation analysis in alkaline sucrose and cesium chloride-ethidium bromide gradients



(Shvedchikova and Tarasevich, 1968; 1971; Summers, 1975; Summers and Anderson, 1972a; 1972b). Two DNA components were observed in these gradients; one component sedimented characteristically as a covalently closed, circular molecule while the other sedimented as a relaxed, circular molecule. Tweeten *et al.* (1977b), have confirmed the superhelical and circular nature of the DNA from *P. interpunctella* GV which has a molecular weight of  $76 \times 10^6$  daltons. The large size of this genome is comparable to the estimates of  $90 \times 10^6$  molecular weight for *S. frugiperda* and *T. ni* GVs (Summers and Anderson, 1972b), as well as other viruses (Brown *et al.*, 1977; Burgess, 1977; Tweeten *et al.*, 1977b; 1980b). Based on sedimentation analysis, reassociation kinetics, electron microscopy and restriction enzyme digestions, the size of the viral genomes ranges from  $69 \times 10^6$  to  $111 \times 10^6$  molecular weight (Brown *et al.*, 1977; Burgess, 1977; Shvedchikova and Tarasevich, 1968; 1971; Tweeten *et al.*, 1977b; 1980; Summers and Anderson, 1972b; 1973; Smith and Summers, 1978). Biophysical parameters such as thermal melting points, and G + C content have been determined for many GV DNAs (Harrap and Payne, 1979; Tweeten *et al.* 1977b).

The use of modern molecular techniques, such as restriction endonuclease cleavage and reassociation kinetics, to analyze the genomes of GVs and NPVs has provided new insights into baculovirus molecular biology. The genomic complexities of some GV DNAs has been measured by such methods, and the data indicate that the GV DNA of *P. brassicae* and *P. interpunctella* (Scharnhorst and Weaver, personal communication) may consist of primarily single copy sequences. In addition, restriction

endonuclease fragmentation analysis indicates that the GVs of S. frugiperda, T. ni and P. rapae and P. interpunctella are not closely related (Tweeten et al., 1980b). This technique has proved useful, however, in the analysis of the genomes of NPVs (Miller and Dawes, 1978a; Rohrmann et al., 1978; Smith and Summers, 1978), particularly in the case of related genomic variants (Lee and Miller, 1978; Miller and Dawes, 1978b). The establishment of genetic maps and relationships to phenotype characteristics has been undertaken with a number of NPV DNAs (Smith and Summers, 1980; Summers et al., 1980). Unfortunately, GV DNAs have not been mapped genetically, and establishing a relationship between such a map and phenotype expression will be difficult since an in vitro culture system for these viruses does not, as yet, exist.

## II. Granulosis Virus Replication.

### In Vivo Replication.

A number of reports have been published on the replication of granulosis viruses within their respective insect hosts. Most of the evidence presented is based on electron microscopic analysis of infected tissues; very little biophysical or biochemical data exists to substantiate these observations.

The replication cycle of GV appears to be far more complex than that observed with vertebrate viruses. The primary organ infected by GV is the fat body which is the major site of intermediary metabolism and storage of fat, protein, glycogen, etc. (Arnott and Smith, 1968a; Asayama and Osaki, 1970; Carner and Barnett, 1975; Hamm and Paschke,

1963; Huger, 1963). Other organs such as the epidermis (Beegle, 1979; David, 1978; Kawanishi et al., 1972b), hemocytes (blood cells) (Stairs, 1966), tracheal matrix cells (Beegle, 1979; Hunter et al., 1975), and the malpighian tubes may be also infected (Hunter et al., 1975; Sato et al., 1972; Stairs et al., 1966), but this has not been demonstrated in all lepidopteran species.

Infection is initiated in the midgut by ingestion of the virus (Summers, 1969; 1971; Tanada and Leutenegger, 1970). The virus appears to go through two stages of infection wherein the midgut cells lining the intestinal lumen support one phase of replication leading to progeny virus that then infects other tissues, principally the fat body. Infection of fat body cells is followed by replication and release of progeny virus that can reinfect other tissue or, later in the cycle, be released in the occluded form.

The first step in the replication cycle involves release of the infectious entity, the enveloped nucleocapsid, from the occluded form. Alkaline conditions in the gut lumen of lepidopteran insects provides the environment necessary to solubilize the protein matrix thereby releasing the EN (Faust and Adams, 1966; Martignoni, 1957). Certainly, the presence of alkaline proteases within the protein matrix would aid dissolution and release of EN, but the role of such enzymes in vivo has not been established (Eppstein et al., 1975; Kozlov et al., 1975; McCarthy and Liu, 1976; Tweeten et al., 1978). The exact conditions of the gut lumen are unknown, and it has been suggested that such factors as synergistic factors or enzymes (Hara et al., 1976; Tanada et al.

1973; 1975; Tanada and Hara, 1975; Yamamoto and Tanada, 1978), or other undefined components may aid in the release and subsequent infection of cells by EN. The midgut dissolution process has been examined in GV-infected larvae of T. ni, the cabbage looper (Summers, 1971). Electron microscopic inspection of gut tissue fixed shortly after the virus was ingested revealed the protein matrix in various stages of disruption. Enveloped nucleocapsids were released as the matrix split open and were found associated with the microvilli of midgut columnar cells. Viral penetration appears to occur via fusion of the envelope with the outer cell membrane thereby releasing the nucleocapsids into the cell cytoplasm (Harrap, 1970; Kawanishi et al., 1972b).

The uncoating mechanism(s) of GV nucleocapsids at the nuclear membrane has been observed by electron microscopy (Summers, 1971). At two to six hours after infection, intact nucleocapsids were observed associated end-on with the nuclear pores with empty or partially empty capsids located outside the nucleus. These observations suggested that the viral DNA is inserted into the nucleus without the capsid structure. Similar events have been reported occurring with infection of cells with NPVs (Kawanishi, 1972b; Raghow and Grace, 1974; Tanada and Hess, 1976).

Approximately 12 to 18 hours post-infection, progeny nucleocapsids can be seen in the midgut nucleus. Interestingly, the nucleus of an infected midgut cell has regions of dense aggregated material which is distinct from host chromatin; the nucleocapsids are observed in these

areas (Hunter et al., 1975; Summers, 1971). Nucleocapsids can acquire membrane from specific regions of the nuclear membrane or, when the integrity of the membrane is lost, can obtain envelope from intracytoplasmic membranes which appear to proliferate during replication (Tanade and Leutenegger, 1970). Additionally, nucleocapsids may bud through regions of the plasma membrane, which have been modified by insertion of peplomeric structures; the "peplomers" are retained as the EN are released into the hemocoel (Robertson et al., 1974). Enveloped nucleocapsids present within the cell appear to be incorporated into vacuoles which are transported to the basal membrane where they are released by some unknown mechanism (Hunter et al., 1975; Robertson et al., 1974; Summers, 1969).

The next phase of the replication cycle involves infection of other larval tissues. It is rare for the EN released from infected midgut cells to be occluded in matrix protein, and it is believed they are responsible for the secondary infection (Harrap et al., 1977). It has been demonstrated that tracheal tissue adjacent to midgut cells is frequently infected, and it is likely that EN released from the midgut may be responsible (Beegle, 1979; Hunter et al., 1975). However, the question of whether or not the viral progeny from midgut cells also infects hemocoelic tissues has not been settled. For example, Tanada and Leutenegger (1970), have suggested that some EN from occluded virus may pass directly from the gut lumen into the hemocoel via intercellular spaces thereby bypassing midgut cell infection.

The fat body is the primary tissue in lepidopteran larvae. As such, the events of secondary infection and replication in fat body cells has been of great interest, and has been studied in several species (Arnott and Smith, 1968a; 1968b; Asayama and Inagaki, 1975; Benz and Wager, 1971; Hunter et al., 1975; Stairs et al., 1966). The sequence of events in the replication of GV in the fat body cells of P. interpunctella has been described by Arnott and Smith (1968a; 1968b). GV replication in fat body differs from that observed in midgut epithelial cells in many respects. The most significant differences are: 1) occlusion of enveloped nucleocapsids takes place in fat body cells. This involves layering of granulin onto the EN to form the protein matrix; 2) many cells are infected with the yield of progeny virus per cell being much higher; 3) unlike midgut cells, fat body cells are lysed which releases the occluded virus into the hemocoel of the insect.

The events associated with initial adsorption and entry of GV enveloped nucleocapsids into fat body cells are not known. Investigations of NPV infection suggest the virions attach to cell membranes via a modified end of the viral envelope (Adams et al., 1977; Hirumi et al., 1976; Kawamoto et al., 1977b). These modifications may be the peplomer-like structures observed on nucleocapsids which budded (Summers and Volkman, 1976). Penetration of the virus into the cell appears to occur through viropexis.

The release of nucleocapsids from the envelope and subsequent uncoating has not been studied in vivo because synchrony of fat body

infection is difficult. The initial event observed by electron microscopy is an increase in nucleus size and the redistribution of host chromatin toward the nuclear membrane. A network of electron dense material resembling aggregated chromatin gathers in the nucleus. This network, referred to as "virogenic stroma" becomes increasingly Feulgen-positive, and the host chromatin degenerates. In P. interpunctella and C. muriana, the hypertrophied nucleus rapidly disintegrates and the stroma mixes with the contents of the cytoplasm such that nucleocapsids are first detected in the cytoplasm (Arnott and Smith, 1968a; 1968b; Huger and Krieg, 1961). However, in C. pomonella, T. ni, P. opercullella, the nucleus remains intact for a longer period of time such that replicated nucleocapsids are observed in the nucleus (Benz and Wager, 1971; Stairs et al., 1966; Summers, 1971). In either case, the virogenic stroma appears to be the site of viral DNA synthesis and nucleocapsid assembly (Xeros, 1956). After the breakdown of the nuclear membrane, nucleocapsid formation may remain in the region formerly occupied by the nucleus (Benz and Water, 1971; Stairs et al., 1966), or may spread throughout the cell (Bird, 1963).

The acquisition of envelope material seems to be a complex and diverse process. With some GV, envelopes are acquired by budding through nuclear or cytoplasmic membranes or are obtained by de novo synthesis (Bird, 1964; Pinnock and Hess, 1978; Stoltz et al., 1973; Summers, 1971). In P. interpunctella, envelopes are obtained from large masses of smooth endoplasmic reticulum that accumulate during replication.

Nucleocapsids align themselves in regular arrays along the length of the reticulum and are inserted into the envelope (Arnott and Smith, 1968a). Similar processes have been observed with the GVs of Plutella xylostella (Asayama, 1975), and Cadra cautella (Hunter and Hoffman, 1970).

The occlusion process begins by deposition of granulin on the surface of the viral envelope at one side or one end of the envelope, and proceeds around the virion (Asayama, 1975; Hunter and Hoffman, 1970; Pinnock and Hess, 1978; Watanabe and Kobayashi, 1970). Only enveloped nucleocapsids have been observed to be occluded in protein matrix, and this occurs solely in fat body cells (Bergold, 1963b).

The role of nonoccluded enveloped nucleocapsids in hemocoelic infection has been established in the A. californica NPV system (Summers and Volkman, 1976). Thus, progeny enveloped nucleocapsids which remain nonoccluded may be involved in infection of other cells; although, nucleocapsids that bud through the plasma membrane could also be involved in spreading the infection.

In the final stages of infection, large masses of cells become packed with GV and eventually rupture liberating GV into the hemocoel (Huger, 1963; Arnott and Smith, 1968a; 1968b). The GV of P. interpunctella appears white due to the opacity of the occluded form, because such large numbers of GV produced, the fat body and hemocoel become milky white. The insect loses its normal pink-brown color and appears white in color. The insect dies soon thereafter.



Knowledge of the biochemical and biophysical basis of the sequence of events described above is limited. Studies of  $^3\text{H}$ -uridine and  $^3\text{H}$ -thymidine uptake in GV-infected C. pomonella have shown there is a sharp rise in RNA synthesis in the cytoplasm, chromatin, and nucleoli of fat body cells prior to any cytopathological changes (Benz and Water, 1971). At 25 hours post-infection, RNA synthesis appears to be localized in swelling nucleoli (Wager and Benz, 1971), after which time the nucleoli degenerate and the levels of RNA synthesis return to normal levels. During nucleoli degeneration, the host chromatin is redistributed to the peripheral regions of the nucleus.

DNA synthesis does not appear to significantly change in the first 24 hours after infection. Although early DNA synthesis has been observed with some GV-infected insects, the highest incorporation of thymidine into DNA occurred concurrently with hypertrophy of the nucleus and formation of the virogenic stroma some 30 to 40 hours post-infection. A second, large increase in label incorporation was seen at the periphery of the virogenic stroma during membrane degeneration (Watanabe and Kobayashi, 1970). At 60 to 70 hours after infection, DNA synthesis was about 30 times that of normal, uninfected cells.

An increase of RNA synthesis accompanied that of DNA and incorporation of label into RNA continued until the enveloped nucleocapsids were occluded. RNA synthesis rapidly decreased once the occlusion process was completed.

Complementary studies on protein synthesis during GV replication in vivo are lacking. Incorporation of  $^3\text{H}$ -tyrosine into newly synthesized

protein associated with virogenic stroma has been reported in the infected fat body cells of Hyphantria cunea (Wantanbe and Kobayashi, 1970).

#### In Vitro Replication.

One of the primary obstacles in studying the biochemical and biophysical characteristics of granulosis viruses is the inability of these viruses to replicate in vitro. Unlike the nuclear polyhedrosis viruses which are propagated successfully in tissue culture (Goodwin et al., 1970), GV's have not been found to infect and fully replicate in insect cell lines in vitro. Established cell lines such as S. frugiperda, T. ni, and H. zea have been examined as possible hosts (Granados, 1976), but successful replication has not been observed. In T. ni cells, an aberrant GV infection occurred in which numerous membrane structures and long cylinders resembling capsids were synthesized, but normal nucleocapsids or enveloped nucleocapsids were not formed nor was infectious progeny produced. Incomplete development of virus has been reported following infection of heterologous insect hosts with GV's and NPV's (Hunter and Hoffman, 1972), or in prolonged serial passage of some NPV's in vitro (MacKinnon et al., 1974).

Infection of primary cultures of L. dispar ovaries by GV has been reported (Vago and Bergoin, 1963). This involved incubation of primary cultures with GV-infected fat body of P. brassicae; infection of the cultures with enveloped nucleocapsids from solubilized, occluded virus was not successful. Unfortunately, virus production was irregular and

incomplete. In only some cultures were nuclear hypertrophy and occlusion of virus observed. Other attempts to infect cultured cells with GV have been made (Granados, 1976), but no information regarding what influence cell species, cell type, nutritional or hormonal supplements, etc. may have on viral replication is available. Additionally, the source of viral inoculum and conditions for absorption and infection may greatly influence the ability to infect cells in vitro. It is of great interest, then, to establish parameters that may affect viral absorption and binding to cells.

## SECTION IV

AGGLUTINATION OF VERTEBRATE ERYTHROCYTES BY THE  
GRANULOSIS VIRUS OF PLODIA INTERPUNCTELLA

### Abstract

The granulosis virus (GV) of the Indian meal moth, Plodia interpunctella (Hübner), was found to agglutinate several species of vertebrate erythrocytes with differential specificity. Optimal conditions for hemagglutination of rabbit cells were established and used to study interactions between GV and vertebrate cells. Electron microscopy and hemagglutination-inhibition studies indicated that aggregated enveloped nucleocapsids were involved in the agglutination phenomenon. Enzymatic treatments of erythrocytes suggested that GV interacted with neuraminidase and trypsin-sensitive cell membrane components. Furthermore, binding studies demonstrated that adsorption of  $^{125}\text{I}$ -GV to cells occurred not only under acidic conditions but also under alkaline conditions where hemagglutination was not observed. It is believed that although GV binds to cells under both acidic and alkaline conditions, hemagglutination is facilitated by aggregation of GV at low pH.

### Introduction

The granulosis and nuclear polyhedrosis viruses constitute a unique class of animal viruses. As members of the family, Baculoviridae, these rod-shaped viruses characteristically infect insect hosts and are being considered for use as biological insecticides (Falcon, 1976). Our laboratory is concerned specifically with the biophysical and biochemical characterization of the granulosis virus (GV) of the Indian meal moth, Plodia interpunctella (Tweeten et al., 1977a; 1977b; 1978; 1980a; 1980b; 1980c), as well as the process(es) of infection by this virus at the cellular and molecular level.

Although much information can be obtained from in vivo infection, a concise investigation of the infection process requires in vitro manipulation of the virus and cells under controlled conditions. Unfortunately, the granulosis viruses as a group do not appear to infect cells in vitro, and no suitable tissue culture system has yet been found to support their replication. Because the first step in the infection process is adsorption of the virion to the membrane of the host cell, the problem of in vitro infection with granulosis viruses could reside in the initial interactions of the virus with the cell i.e., conditions and mechanism of adsorption to the host cell. In several cases, the adsorption of viruses to cells has been mediated through the same receptors that are involved with agglutination of vertebrate erythrocytes (Howe and Lee, 1970; Bolen and Consigli, 1979). It is our belief that the hemagglutination phenomenon is useful for investigating the mechanism of viral adsorption to potential

host cells. Therefore, we have examined the ability of Plodia GV to hemagglutinate cells.

Hemagglutination of erythrocytes by several insect viruses has been reported (Cunningham et al, 1966). A cytoplasmic polyhedrosis virus (CPV) and nuclear polyhedrosis virus (NPV) of Bombyx mori both have been shown to agglutinate sheep, chicken, and mouse erythrocytes (Miyajima and Kawase, 1969), and Suto and Kawase (1971) reported a non-occluded Flacherie virus of B. mori that agglutinates mouse cells. Shapiro and Ignoffo (1970) found that the NPV of Heliothis zea agglutinates chicken erythrocytes and demonstrated that the virions were responsible for hemagglutination. Reichelderfer (1974) and Norton and DiCapua (1978) found that the polyhedrin of Spodoptera frugiperda NPV and Lymantria dispar NPV also hemagglutinated chicken erythrocytes. To date, there has been no report of a granulosis virus agglutinating vertebrate erythrocytes. In the present study, we utilized the hemagglutination of erythrocytes as a model to investigate the adsorption process associated with Plodia interpunctella GV. The data presented in this paper demonstrate that enveloped nucleocapsids of GV are capable of agglutination of erythrocytes, that specific interactions between virion and cell surface components are sensitive to neuraminidase and trypsin, and that the virus is capable of binding to cells under both acidic and alkaline conditions.

## Materials and Methods

### Preparation and purification of GV

GV was produced in a laboratory colony of P. interpunctella (Hübner) reared as previously described (Tweeten et al., 1977a). Early third instar larvae were infected per os with GV, and the virus was purified by differential centrifugation, treatment with 1% deoxycholate, and velocity sedimentation in sucrose gradients (Tweeten et al., 1977a; Tweeten et al., 1978).

### Preparation of hemagglutinin (GV-PRH) from purified GV

Suspensions of GV were treated by a modified method of Reichelderfer (1974). Aliquots of purified, occluded GV were pelleted at 15,000 RPM in a Sorvall RC-2B centrifuge with SS 34 rotor for 30 min and were resuspended in one-fourth the original volume with carbonate buffer (0.03 M  $\text{Na}_2\text{CO}_3$ , 0.02 M NaCl, pH 11.0). Exposure of the occluded virus to alkaline conditions in vitro activated an alkaline protease within the protein matrix that digested the matrix to the granulin monomer. In all but the alkaline exposure experiments, solubilization of the protein matrix proceeded for 60 min at 22° with constant stirring. For alkaline exposure experiments, preparations of GV were exposed to alkaline conditions for either 5 or 60 min at 22°. The protease was inactivated and solubilization stopped by lowering the pH by adding seven volumes of borate-saline buffer, pH 8.0 (BSB; 0.12 M NaCl, 0.05 M  $\text{H}_3\text{BO}_3$ , 0.098 M NaOH) and mixing at 22° for 10 min. The suspension was then centrifuged at 34,000 RPM in a SW 41 rotor for 120 min at 10°. The pellet containing aggregated enveloped nucleo-



capsids (GV-PRH) was washed once with BSB, resuspended in 0.01 M Tris-HCl, pH 7.5, at 1/20 original volume and stored at 4° for a maximum of one week. The appearance of aggregated virions in the GV-PRH preparation, compared with free-enveloped nucleocapsids, is shown in Fig. 5. Protein determinations were made by using the Coomassie brilliant blue color assay of Bradford (1976) as specified by Biorad Laboratories. Bovine serum albumin and lysozyme were used as protein standards. The protein content of GV-PRH ranged from 200 to 500 µg/ml depending on the preparation. In all experiments, single preparations of GV-PRH were used to rule out variation of hemagglutination activity due to protein concentration.

#### Hemagglutination (HA) assay

Serial two-fold dilutions of 0.025 ml of GV-PRH were made in microtiter plates (Microbiological Associates) by using 0.03 M  $\text{KH}_2\text{PO}_4$ - $\text{Na}_2\text{HPO}_4$  buffered saline (K- $\text{Na}_2\text{PBS}$ ) as diluent (Fetjenu, 1978). A 0.05-ml aliquot of a 0.75% suspension of washed erythrocytes in buffer of desired pH was added to each well, agitated briefly, and incubated at 25° for 120 min. Hemagglutination activity was determined as the reciprocal of the highest two-fold dilution that gave complete agglutination. The pH values expressed in the text and the figures represent the final pH of each diluent-hemagglutinin mixture.

Several species of erythrocytes were used in the assay. Chicken, sheep, cow and rabbit cells in Alsever's solution were commercially obtained (Colorado Serum Co.). In addition, rabbit, guinea pig, and human AB-Rh<sup>+</sup> and O-Rh<sup>+</sup> cells were privately obtained. Cells privately

obtained were washed once in phosphate-buffered saline (PBS), pH 7.2, resuspended in sterile Alsever's solution, and stored at 5° for a maximum of five days. Prior to use, all cells were washed three times in PBS, pH 7.2, and resuspended to a final concentration of 0.75% in PBS, pH 7.2. For use in the assay, aliquots of erythrocytes were gently pelleted by centrifugation and resuspended in an equal volume of K-Na<sub>2</sub>PBS of a specific pH. Cells were used within 48 hr after washing.

Optimal conditions for hemagglutination of rabbit erythrocytes were determined by varying the pH, buffer molarity, and incubation temperature as indicated in the figure legend. Buffer molarity and pH were adjusted by varying the molar ratio of KH<sub>2</sub>PO<sub>4</sub> to Na<sub>2</sub>HPO<sub>4</sub> at each temperature used.

The effect of divalent cations on hemagglutination of rabbit erythrocytes by GV-PRH was examined by adding various concentrations of divalent cations to diluent buffer. Because of the chelating nature of phosphate buffers, 0.02 M piperazine-N,N'-bis (2-ethane-sulfonic acid), monosodium salt (PIPES), pH 6.6, was used. Various concentrations of CaCl<sub>2</sub>, MgCl<sub>2</sub>, and MnCl<sub>2</sub> were used and the concentration of NaCl was adjusted to yield the correct osmolarity in the presence of each cation. Rabbit erythrocytes resuspended in each buffer solution were examined for adverse reactions to the PIPES and/or cations prior to use in the assay.

#### Hemagglutination inhibition (HAI) assay using specific antisera to GV components

Antisera to occluded GV, purified granulin, enveloped nucleocapsids

and nucleocapsids (N) were generated in New Zealand white rabbits. Purified occluded GV, EN and N were prepared as described (Tweeten et al., 1977a; 1978; 1980c). Granulin was obtained from carbonate treated GV and subsequently isolated from sodium dodecyl sulfate-polyacrylamide tube gels (SDS-PAGE) and prepared as described previously (McMillen and Consigli, 1977). All antigen preparations were emulsified in Freund's complete adjuvant (for primary injection only) or incomplete adjuvant and injected intramuscularly during the first two weeks and then subcutaneously on the fourth and sixth weeks. Each rabbit received approximately 10 to 50  $\mu$ g of total protein per injection. Rabbits were bled five days after the last injection, the sera extracted and IgG purified by polyethylene glycol (PEG 6000) precipitation (Carter and Boyd, 1979), followed by chromatography on DEAE-Affi-gel Blue matrix (Bio-Rad Labs) in 0.02  $M$   $K_2HPO_4$ , pH 8.0. Preparations of IgG to be used in HAI assays were adjusted to 1 mg/ml and 0.1 mg/ml with 0.01  $M$  PBS, pH 7.2. The reactivity of each antibody preparation was tested by agar-gel immunodiffusion (AGID) which demonstrated that: 1) antibody to the occluded virus would react with purified granulin, enveloped nucleocapsid, and nucleocapsid proteins; 2) antibody to EN would react with enveloped nucleocapsid or nucleocapsid proteins but not with granulin; 3) and antibody to nucleocapsids would react with nucleocapsid proteins.

HAI assays were performed by making serial two-fold dilutions of a 0.025 ml volume of antibody at 1.0 mg/ml or 0.1 mg/ml IgG ranging from 1:2 through 1:256 in 0.03  $M$   $K-Na_2$ PBS, pH 6.6. An equal volume of 16 HA units of GV-PRH was added to each well and incubated at 25° for 120 min.

A 0.05 ml aliquot of 0.75% suspension of rabbit erythrocytes in pH 6.6 buffer was added, agitated, and incubated for an additional 120 min. Controls consisted of: 1) 0.025 ml of the IgG preparation of interest mixed with 0.025 ml of diluent; 2) preimmune IgG mixed with 0.025 ml of 16 HA units of GV-PRH; 3) 0.025 ml of diluent mixed with 0.025 ml of 16 HA units of GV-PRH; 4) diluent alone as the negative HA control. HAI titer of the antibody was determined as the reciprocal of the highest dilution of IgG giving complete inhibition of hemagglutination.

#### Enzymatic treatment of erythrocytes

Rabbit erythrocytes were treated with neuraminidase, trypsin and hyaluronidase prior to use in hemagglutination assays. For each enzymatic treatment, a 0.75% suspension of cells was centrifuged to a packed volume of 0.4 ml. A total of 300 or 500 units of neuraminidase (Calbiochem; V. cholerae, protease, aldolase, lecithinase C and  $\beta$ -galactosidase-free) was added to the packed cells, and the volume brought to 2.0 ml with 0.01 M PBS, pH 7.2. The cells were incubated at 37° for 60 min. Trypsin treatment of cells followed the method of Morton and Pickles (1951) and Cook et al. (1960), in which four times the packed volume of cells of a 50 or 500  $\mu$ g/ml solution of bovine pancreas trypsin (2X recrystallized, tissue culture grade, USA Biochemicals) was added to the cells and incubated at 37° for 30 min. A total of 100 units of streptococcal hyaluronidase (Calbiochem) in 0.05 M PBS, pH 6.0 was added to cells. The suspension was brought to a final volume of 2.0 ml and incubated at 37° for 60 min. All treated cells were washed three times by centrifugation and resuspended to a final con-

centration of 0.75% in PBS, pH 7.2. All treated cells were used immediately. Polyoma virus, Newcastle disease virus, and Sendai virus were used as internal controls with enzymatically treated cells.

#### Binding assay with rabbit erythrocytes and radiolabelled GV enveloped nucleocapsids

Gradient-purified enveloped nucleocapsids were surface labeled with  $^{125}\text{I}$  (ICN Biochemicals) using Enzymobead reagent (Biorad) according to Tweeten et al. (1980c). The method of Fries and Helenius (1979) was used to measure binding activity. A 22  $\mu\text{l}$  aliquot of  $^{125}\text{I}$ -EN (specific activity of 42,000 cpm/ $\mu\text{g}$  protein) was added to 1.8 ml of a 0.75% suspension of rabbit erythrocytes. The  $^{125}\text{I}$ -EN were incubated in the presence of cells at 22° or 4° for a total of 360 min with the buffer pH ranging from 6.2 to 8.0. At specified intervals, 0.2 ml of cells was removed, washed three times with buffer of the corresponding pH and aliquots were counted by liquid scintillation spectrophotometry in a Beckman LSC-250 counter. Background counts were determined for each pH by incubating virus in buffer without cells and removing corresponding aliquots at the specified times.

#### Electron microscopy

EN were prepared for electron microscopy as described by Tweeten et al. (1976). Samples were placed on carbon-coated grids and stained with 2% uranyl acetate. Grids were examined on a Philips EM201 at 60KV.

## Results

### Species specificity of hemagglutination by GV-PRH

The hemagglutination activity of GV-PRH was found to vary greatly, depending the species of vertebrate erythrocytes. The data presented in Fig. 1 demonstrate that of the six animal species tested, rabbit erythrocytes were the most sensitive to hemagglutination by GV-PRH whereas bovine cells were the least sensitive. It should be noted that in all cases, optimal hemagglutination occurred under acidic conditions with each species of erythrocyte having a different pH optimum. Rabbit cells showed the greatest activity in the pH range 6.4 - 6.7.

Human AB-Rh<sup>+</sup> and O-Rh<sup>+</sup> cells also were tested for hemagglutination activity. In Fig. 1 (inset), human AB-Rh<sup>+</sup> cells were found to be as sensitive to hemagglutination by GV-PRH as were rabbit cells, and the optimal pH for human AB-Rh<sup>+</sup> cells coincided with that of the rabbit cells. However, human O-Rh<sup>+</sup> cells had negligible reactivity to GV-PRH regardless of the pH (pH 6.2 to pH 8.0). Because of accessibility and sensitivity, rabbit erythrocytes were used throughout this investigation.

### Optimal conditions for hemagglutination

In preparing GV-PRH, we found that time of exposure to carbonate buffer necessary to disrupt the protein matrix affected hemagglutination activity, as did the pH of the borate-saline (BSB) buffer. The hemagglutination activity decreased 8 to 16-fold when GV was exposed to carbonate buffer for 5 min rather than 60 min. Exposure to the active protease under alkaline conditions for longer than 60 min was avoided

since damage to the envelope and envelope proteins occurred (Tweeten et al., 1978; Wood, 1980). If the pH of the buffer was reduced from pH 8.0 to pH 7.6, hemagglutination activity decreased 256 to 512-fold.

Optimal reaction conditions for hemagglutination were determined by examining the effect of pH, buffer molarity, and temperature of incubation on the interaction between GV-PRH and rabbit erythrocytes. As shown in Fig. 2, greatest activity was observed with 0.03 M K-Na<sub>2</sub>PBS at pH 6.6 and 25°. Although the same level of HA activity was observed at molar concentrations greater than 0.03 M K-Na<sub>2</sub>PBS and temperatures higher than 25°, the erythrocytes lysed readily and endpoints were difficult to establish. Thus, the standard assay conditions were established as 0.03 M K-Na<sub>2</sub>PBS at pH 6.6 and 25°.

The data presented in Fig. 3 demonstrate the effect that divalent cations had on HA activity. Because phosphate buffers ordinarily chelate various cations, we used 0.02 M PIPES buffer rather than phosphate buffer. Control experiments indicated that the PIPES buffer had no detrimental effects on erythrocytes (data not shown). Hemagglutination activity was enhanced two-fold in the presence of 0.01 M Mn<sup>+2</sup> and Ca<sup>+2</sup>. There was no effect on HA activity at concentrations of 0.05-0.15 M Ca<sup>+2</sup> or 0.01 M Mg<sup>+2</sup>; however, with Mg<sup>+2</sup> at concentrations from 0.05 M to 0.2 M, the activity decreased two to four-fold. We did find that, depending on the concentration, several cations caused nonspecific aggregation of cells. For example, Zn<sup>+2</sup> at any concentration, Ca<sup>+2</sup> at 0.2 M and above, or Mn<sup>+2</sup> at 0.05 M and above could not be used.

#### Hemagglutination inhibition by specific antibodies to GV

To determine which component of the occluded virus was responsible for hemagglutination, inhibition (HAI) assays were employed by using specific antisera to occluded GV, granulin, enveloped nucleocapsids or nucleocapsids. The data presented in Table 1 indicate that at either of the antibody concentrations (1 or 0.1 mg/ml), anti-EN IgG gave the greatest inhibition of hemagglutination activity. Anti-EN IgG was capable of inhibiting 16 HA units of GV-PRH by factors of 128 and 64 times, as compared to anti-occluded GV and anti-nucleocapsid IgG, respectively. Similar results were obtained when 8 HA units of GV-PRH were used. In both cases, anti-N or anti-occluded GV IgG could inhibit HA only to a small degree as compared with anti-EN IgG. Anti-granulin IgG did not inhibit HA at any concentration.

The HAI data suggested that enveloped nucleocapsids were involved in hemagglutination. To further substantiate these data, purified enveloped nucleocapsids, nucleocapsids and occluded virus were tested for hemagglutination activity. Purified EN, N and occluded virus were exposed to either acidic or alkaline pH conditions so as to cause aggregation similar to that seen with GV-PRH. Hemagglutination was observed only with aggregated EN (data not shown).

#### Sensitivity of hemagglutination to enzymatic treatment of erythrocytes

The specificity of hemagglutination was studied by treating erythrocytes with neuraminidase, trypsin, and hyaluronidase to remove glycoproteins or sialic acid residues that may be necessary for adherence of the virus to the cell surface (Table 2). Newcastle disease virus and



Sendai virus representing examples of enveloped hemagglutinating virus were intended to serve as positive controls; however, rabbit erythrocytes were not agglutinated by either of these viruses. Polyoma virus has been found to hemagglutinate erythrocytes via sialic acid residues (Bolen and Consigli, 1979), so instead that virus was used as a positive control for the enzyme treatments. Neuraminidase and trypsin treatment of the cells completely abolished hemagglutination by GV-PRH and polyoma virus as compared with untreated cells; however, hyaluronidase treatment appeared to enhance the activity of both viruses. Presumably, HA by GV-PRH occurs via specific erythrocyte glycoproteins that are sensitive to trypsin or neuraminidase. In preliminary experiments, inositol was found to inhibit hemagglutination of rabbit erythrocytes (data not shown). Because the enzymatic treatments eliminated hemagglutination, we suspect that the interaction between GV-PRH and the cells was not due to lipid-lipid interactions but was mediated via specific receptors on the cell membrane.

Binding of  $^{125}\text{I}$ -labeled enveloped nucleocapsids to rabbit erythrocytes.

The binding of purified EN to rabbit erythrocytes was examined by incubating  $^{125}\text{I}$  - EN with cells under different pH conditions. As shown in Fig. 4A, increasing amounts of viral-associated radioactivity were associated with the cells with time. The highest levels of binding were observed at pH 6.6 and pH 7.6. The first peak of binding activity at pH 6.6 correlated with the same pH where optimal hemagglutination was observed. The binding activity at pH 7.6, however, occurred under conditions in which there was no hemagglutination. Binding activity

appeared to decrease to background levels at pH 6.8, which closely paralleled that of hemagglutination activity, i.e., once the optimal pH was reached, the activity dropped rapidly (Fig. 1). The kinetics of binding of EN to rabbit erythrocytes is shown in Fig. 4B. These data represent total binding at pH 6.6 and pH 7.6 during a six-hour period. Although initial binding was higher at pH 7.6 than at pH 6.6, the binding appeared saturable, with both curves reaching approximately the same level after several hours of incubation. Similar results were observed when EN were incubated with cells at 4°. Thus, enveloped nucleocapsids bound to cells whether the pH was acidic or alkaline.

We observed, by electron microscopy, that the virions or EN were not aggregated between pH 7.4 and 7.8 (Fig. 5B). However, under acidic conditions necessary for HA (pH 6.6), the enveloped nucleocapsids were aggregated into large complexes (Fig. 5A) like that found in GV-PRH. These aggregates did not dissociate when the pH was raised to pH 7.6; yet, hemagglutination was found to be reversible (data not shown). Apparently, hemagglutination was influenced more by the effect that pH had on the physical structure of the virions or cells or both than by differences in adsorption of the virus to the cell, in that binding was observed under both acidic and alkaline conditions.

### Discussion

The agglutination of vertebrate erythrocytes by viruses has been well documented (Howard and Lee, 1970). Although some viruses will hemagglutinate over a large pH range (5.0 to 8.0), agglutination at low

pH is characteristic of the rhabdoviruses, togaviruses, rubella, mumps and mouse leukemia viruses (Howe and Lee, 1970). In addition, insect viruses such as S. frugiperda and L. dispar NPVs will hemagglutinate under acidic conditions (Reichelderfer, 1974; Norton and DiCapua, 1975). The granulosis virus of P. interpunctella also was found to agglutinate various species of erythrocytes below pH 7.0 (Fig. 1).

There is, however, substantial disagreement concerning which components of baculoviruses are responsible for hemagglutination. Agglutination of chicken erythrocytes by H. zea NPV was observed only when virions were present in the preparation; hemagglutination was not observed with soluble polyhedrin protein. Furthermore, hemagglutination could be inhibited only by antiserum to Heliothis virions (Shapiro and Ignoffo, 1970). Reichelderfer (1974) and Peters and DiCapua (1978) reported EN in a highly aggregated state (data not shown). Hemagglutination inhibition assays confirmed that EN were intimately involved in agglutinating erythrocytes (Table 1), and in this respect, our findings agree with that observed for H. zea NPV.

A significant amount of work has been directed toward understanding the mechanisms by which viruses bind to cells and initiate the infectious process (Linser et al., 1977; Bishayee et al., 1978; Fries and Helenius, 1979; Bolen and Consigli, 1979). Binding studies with GV enveloped nucleocapsids were undertaken to determine whether the lack of hemagglutination activity at nonacidic pH was a result of the virions inability to bind to the cells. Our data indicate that virions adsorb to rabbit cells under both acidic and alkaline conditions with maximal

activity seen at both acid and alkaline pH (Fig. 4). Thus, the lack of hemagglutination under nonacidic conditions is probably due to factors other than adsorption to the cell surface.

It appears that aggregation of enveloped nucleocapsids is one factor necessary for hemagglutination. We found that under pH conditions where aggregation did not occur (pH 7.6), hemagglutination was not observed even though enveloped nucleocapsids were bound to erythrocytes (Fig. 1, Fig. 4A). Hemagglutination was observed only with preparations of enveloped nucleocapsids that were aggregated by exposure to pH conditions outside the range pH 7.4-7.8. The role of aggregation in hemagglutination has been investigated in other viral systems. Peters and DiCapua (1978) found that aggregation of the polyhedrin hemagglutinin of L. dispar NPV effectively increased activity due to the relative ease with which aggregated molecules of large molecular weight bridge erythrocytes. Aggregation has also been observed to be a factor in hemagglutination by adenovirus fiber antigens (Howe and Lee, 1970). When isolated from the capsids, the fiber antigens are incomplete hemagglutinins which, although monovalent in binding character, are capable of binding to cells. If these fibers are aggregated, they form multivalent structures capable of agglutinating cells. The adenovirus model also suggests that a structure which is monovalent in binding character can be made multivalent by aggregation. The aggregation of enveloped nucleocapsids may provide the means to convert monovalent binding structures into large multivalent complexes capable of bridging cells.

The effect of pH on the physical nature of the cell is probably another important factor in hemagglutination. We found that once the virions aggregated, they did not dissociate when the pH was either raised or lowered. Yet, hemagglutination was not observed under alkaline conditions even though the aggregate was intact and capable of binding to the cells (Fig. 1). A plausible explanation may be that increased electrostatic repulsion between cells at alkaline pH could prevent hemagglutination even though the virus had adsorbed to the cells (Peters and DiCapua, 1978). To this end we found hemagglutination was a reversible process whenever the pH was raised or lowered.

Knowledge of the receptor site for a viral hemagglutinin would be advantageous in understanding cell-virus interactions. Our studies of the GV hemagglutinin indicate that the virus agglutinates vertebrate erythrocytes via cell surface constituents sensitive to neuraminidase and trypsin (Table 2). Although Peters and DiCapua (1978) demonstrated that n-acetyl neuraminic acid (NANA) residues were not involved in binding of the L. dispar NPV hemagglutinin, there is substantial evidence that sialic acid or NANA residues are prime factors in binding and agglutination of erythrocytes by viruses (Howe and Lee, 1970; Bolen and Consigli, 1979). Furthermore, it is probable that the "receptor site" for Plodia GV enveloped nucleocapsids requires additional carbohydrate constituents. We found that inositol inhibited the hemagglutinating activity of GV, suggesting that inositol may be part of the complete receptor (data not shown). This situation is not unprecedented, because it has been observed with influenza virus that complete removal of NANA

does not totally abolish hemagglutination. Presumably, the proximal portions of the oligosaccharide side chains on the peptide backbone are involved in the interaction (Howe and Lee, 1970).

The present study has demonstrated that an insect virus can adsorb to many vertebrate cells with differential specificity. The fact that such viruses have the ability to cross species boundaries with the potential to infect the cells of these species points out the need to examine these viruses more closely. Moreover, the use of baculoviruses as biological control agents necessitates further investigation of the interactions between these viruses and non-target cells. Such studies are currently underway in our laboratory.

Table 1. Hemagglutination inhibition by specific antisera to granulosis virus components.

<u>Antibody to</u> <sup>b</sup>	Hemagglutination inhibition activity <sup>a</sup>	
	<u>1.00 mg/ml IgG</u>	<u>0.10 mg/ml IgG</u>
Occluded GV	2	0
Enveloped nucleocapsids	256	16
Nucleocapsids	4	0
Granulin	0	0

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<sup>a</sup> Hemagglutination inhibition activity determined as the reciprocal of the highest dilution of IgG giving complete inhibition of 16 HA units (93.8  $\mu$ g protein) of GV-PRH. Similar results were obtained with 8 HA units of GV-PRH.

<sup>b</sup> Serial two-fold dilutions of antibody (at 1.0 mg/ml or 0.1 mg/ml IgG) through 1:512 in 0.03 M  $\text{KH}_2\text{PO}_4$ - $\text{Na}_2\text{HPO}_4$  buffered saline, pH 6.6

Table 2. Effects of enzymatic treatment of rabbit erythrocytes on hemagglutination by GV-PRH.

Viral preparations <sup>a</sup>	Untreated cells <sup>c</sup>	HEMAGGLUTINATION ACTIVITY		
		ENZYME TREATMENT		
		Neuraminidase <sup>d</sup>	Trypsin <sup>e</sup>	Hyaluronidase <sup>f</sup>
GV-PRH <sup>b</sup>	1024	0	0	2048
Polyoma	3200	0	0	6400
Sendai	0	0	0	0
Newcastle Disease	0	0	0	0

<sup>a</sup> Examples of enveloped and non-enveloped hemagglutinating viruses were used as control. Preparations included polyoma virus at 512,000 HA/ml, Sendai virus at 4,000 HA/ml and Newcastle Disease virus at 1600 HA/ml.

<sup>b</sup> GV-PRH had a HA titer of 1024 HA/ml at 312 µg/ml.

<sup>c</sup> Cells treated in same manner but lacking enzymes

<sup>d</sup> HA remaining after treatment with 150 or 250 units/ml neuraminidase for 60 min. at 37°.

<sup>e</sup> HA remaining after treatment with 127.7 or 1277 N.F. units/ml of bovine trypsin for 60 min. at 37°.

<sup>f</sup> HA remaining after treatment with 50 units/ml streptococcal hyaluronidase for 60 min. at 37°.

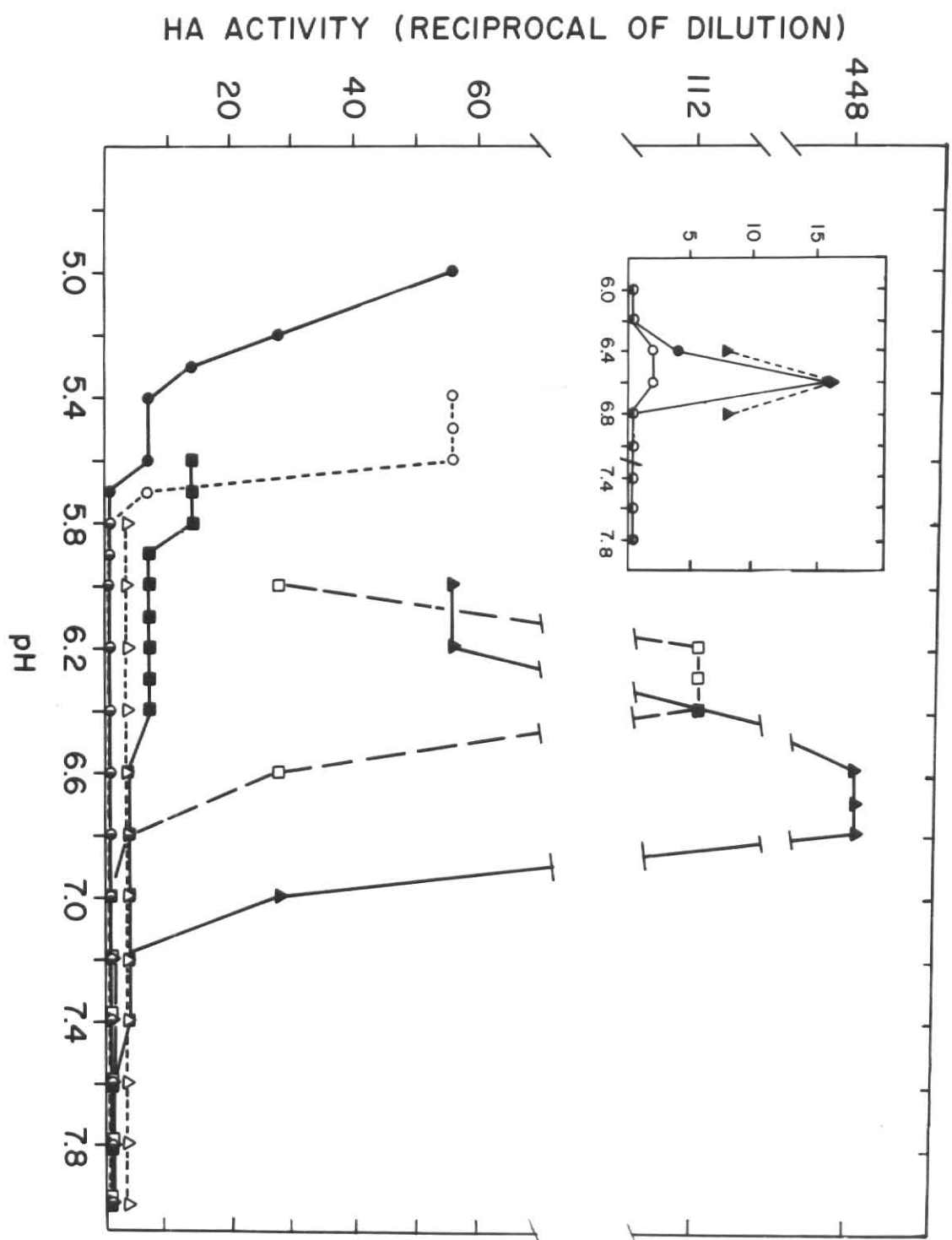


FIGURE 1.

Effect of pH and erythrocyte species on hemagglutination activity.

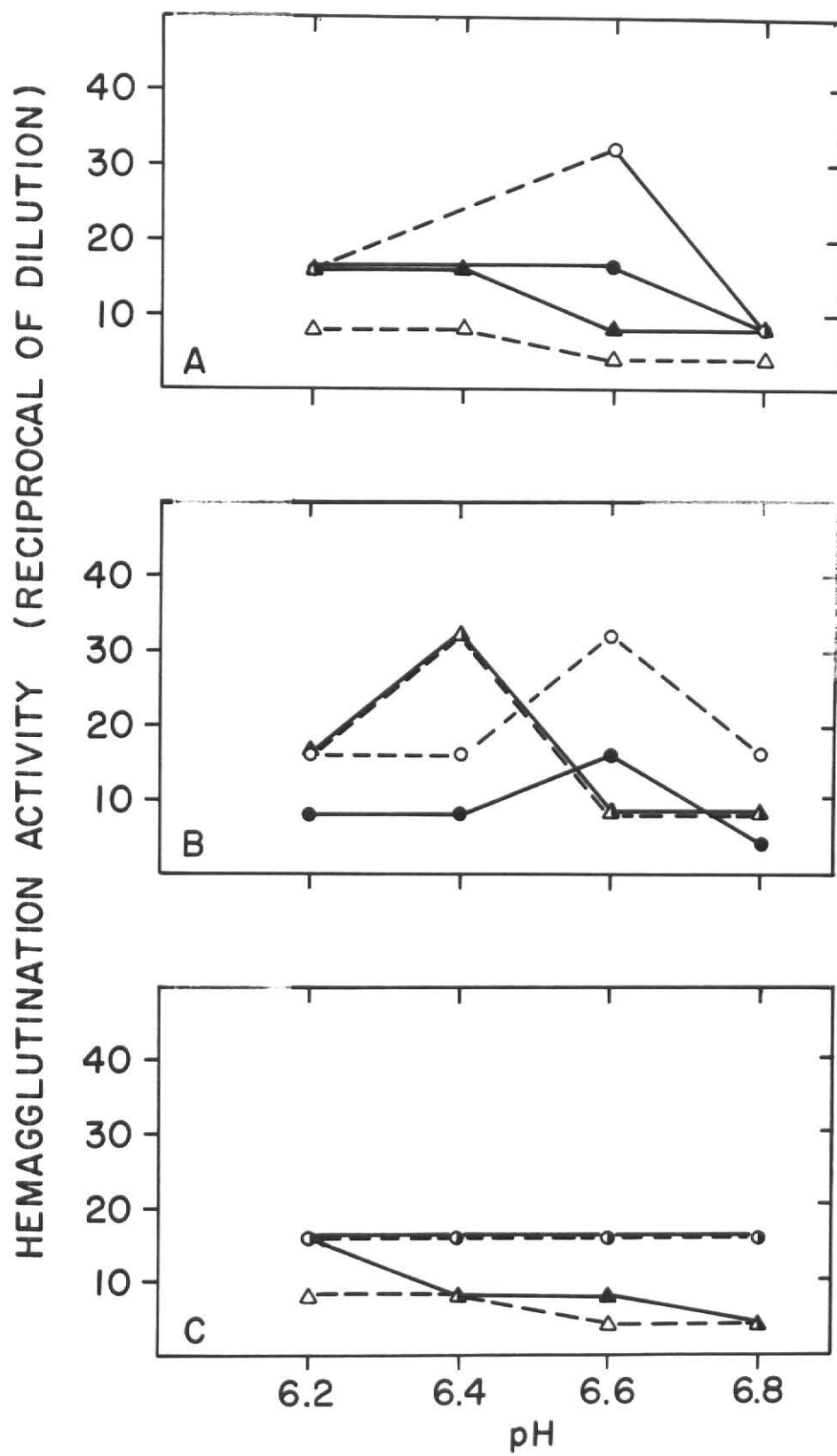
Serial two-fold dilutions of GV-PRH were made in microtiter plates by using K-Na<sub>2</sub>PBS as diluent. The pH varied from 5.0 to 8.0 depending on the species of erythrocyte. Dilutions of GV-PRH ranged from 1:3.5 through 1:7168 with the last well being used as a negative control.

Aliquots of a 0.75% suspension of chicken (●—●), guinea pig (■—■), mouse (□—□), rabbit (▲—▲), cow (△—△), or sheep (○—○) erythrocytes were added to each well and incubated as in Materials and Methods. The protein concentration of GV-PRH used was 402 µg/ml. Inset Aliquots of a 0.75% suspension of human AB-Rh<sup>+</sup> (●—●), human O-Rh<sup>+</sup> (○—○), and rabbit (▲—▲) erythrocytes were added to each well and incubated as above. Dilutions ranged from 1:2 through 1:2048. Protein concentration of GV-PRH used was 50 µg/ml.



## FIGURE 2.

Optimal conditions for hemagglutination of rabbit erythrocytes: buffer molarity, pH, and temperature. (A) Serial dilutions of GV-PRH were made in the following concentrations of buffer: 0.01 M (●●), 0.03 M (○○), 0.6 M (▲▲), 0.1 M (△--△) K-Na<sub>2</sub>PBS at pH (6.2, 6.4, 6.6, and 6.8), as described in Materials and Methods. An aliquot of a 0.75% suspension of rabbit erythrocytes in corresponding buffer was added to each well and incubated for 120 min. at 37°. The titer was determined as the reciprocal of the highest dilution giving complete hemagglutination. (B) Same as in A except incubated at 25°. (C) Same as in A or B except incubated at 4°.



## FIGURE 3.

Effect of divalent cations on hemagglutination of rabbit erythrocytes. Various concentrations of  $\text{CaCl}_2$  (■),  $\text{MgCl}_2$  (□), and  $\text{MnCl}_2$  (▨) were added to 0.02 M PIPES, pH 6.6, which was used as the diluent when serial dilutions of GV-PRH were prepared as described in Materials and Methods. Incubation was at 25° for 120 min. The hemagglutination titer at each cation concentration was determined as described previously.

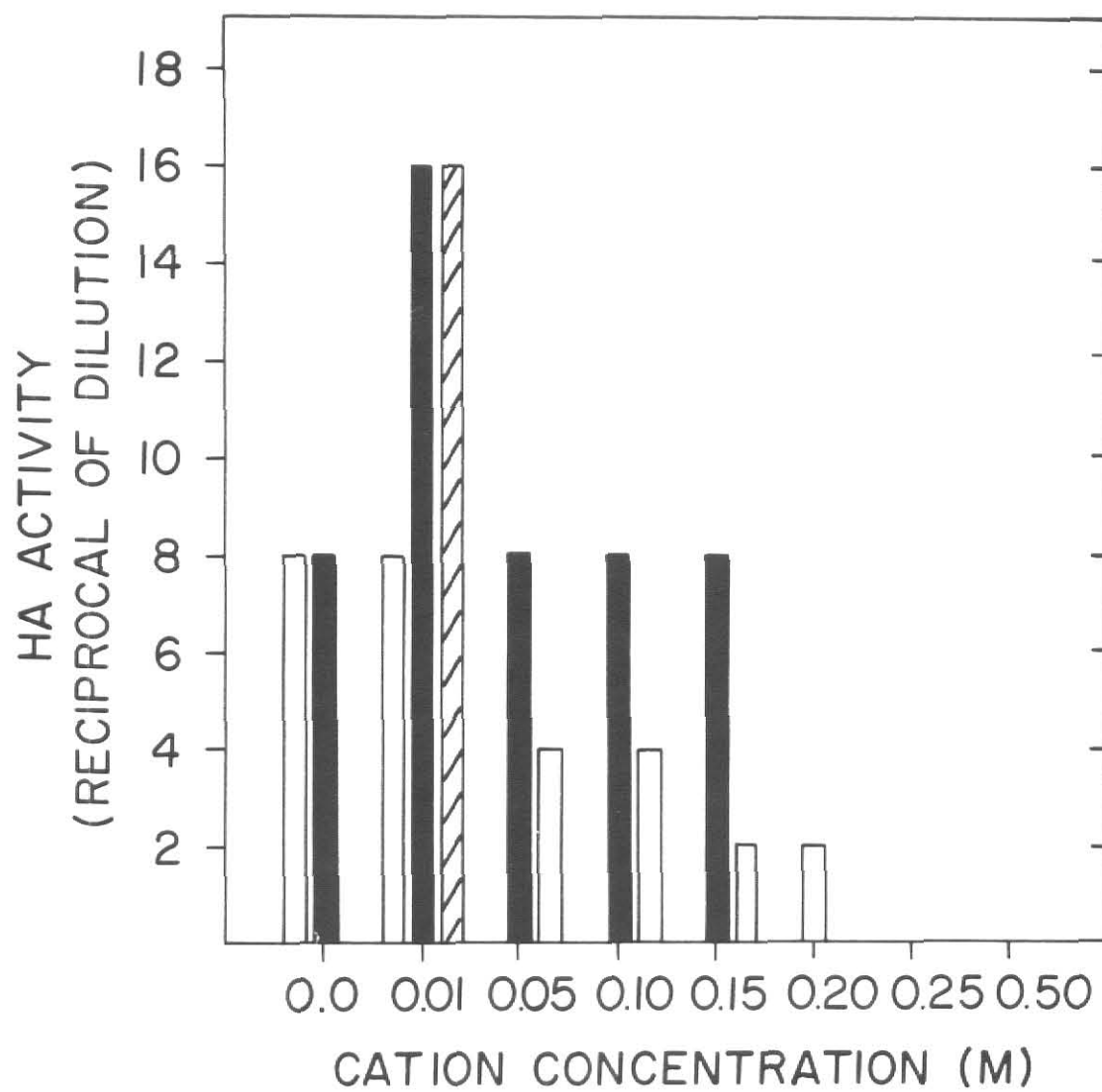
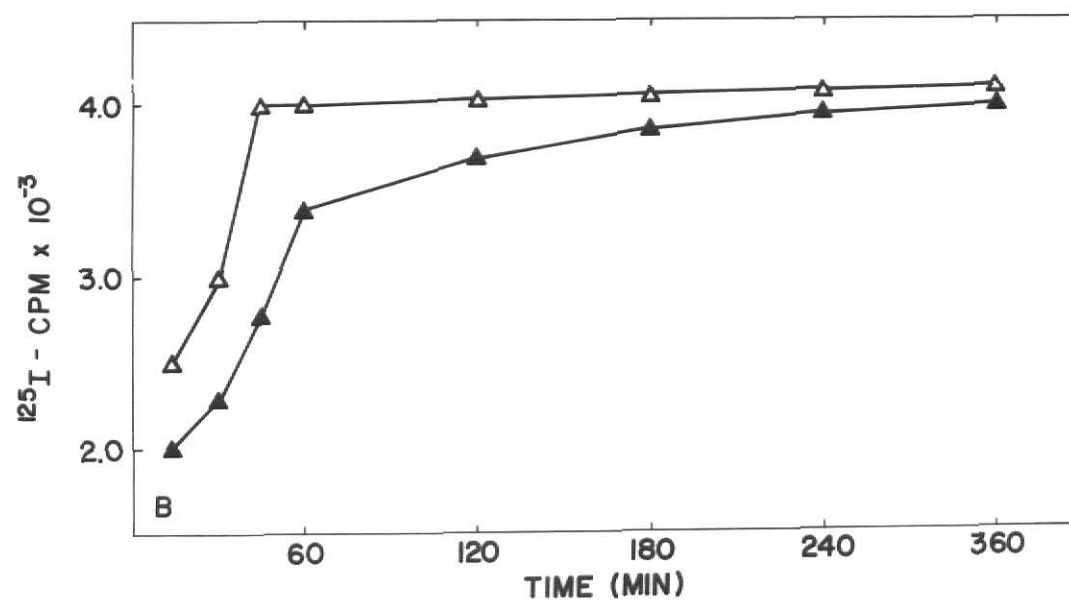
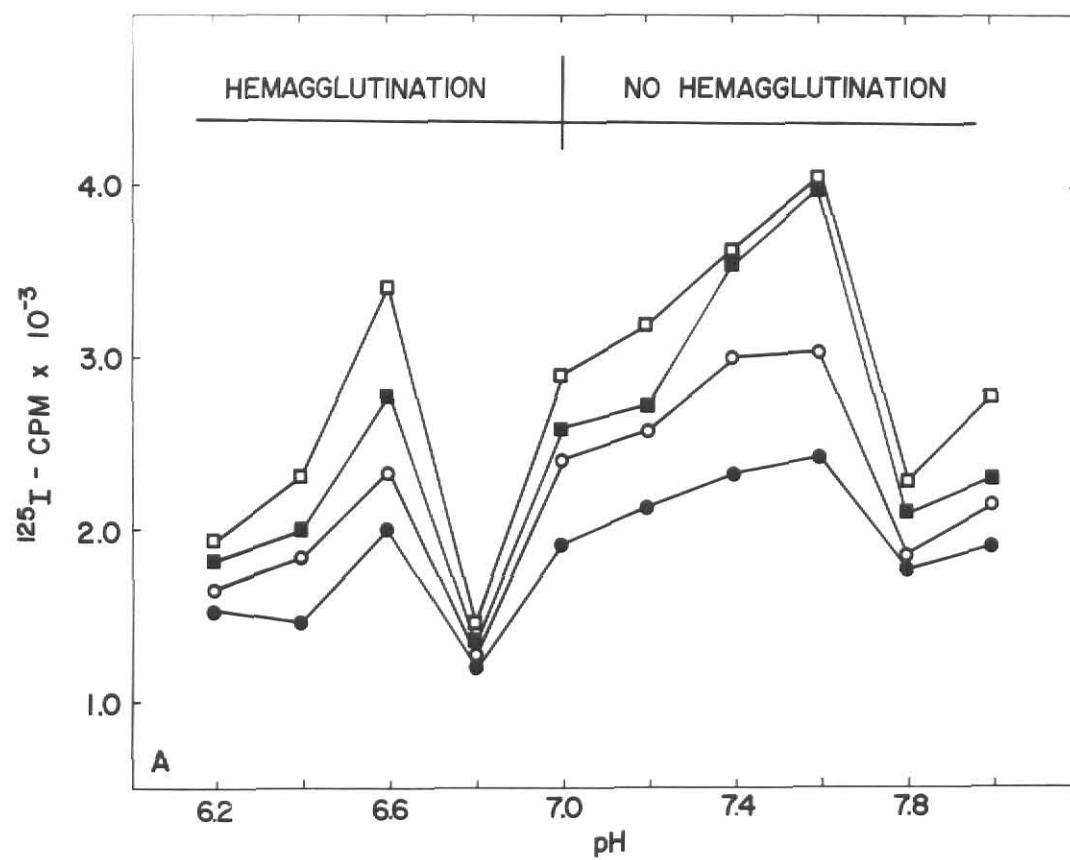


FIGURE 4.

Binding of  $^{125}\text{I}$ -enveloped nucleocapsids to rabbit erythrocytes. Gradient-purified enveloped nucleocapsids were surface labeled with  $^{125}\text{I}$  and aliquots of labelled virus added to a suspension of erythrocytes in  $\text{K-Na}_2$  PBS buffer with pH ranging from 6.2 to 8.0. All tubes were incubated at  $25^\circ$ . At specified intervals, an aliquot of cells was removed, washed with buffer of corresponding pH, and counted by liquid scintillation spectrophotometry. (A) Total binding observed with respect to pH. ●—● 15 minutes, ○—○ 30 minutes, ■—■ 45 minutes, □—□ 60 minutes. (B) Total binding observed at pH 6.6 ▲—▲ and pH 7.6 △—△ with respect to time.





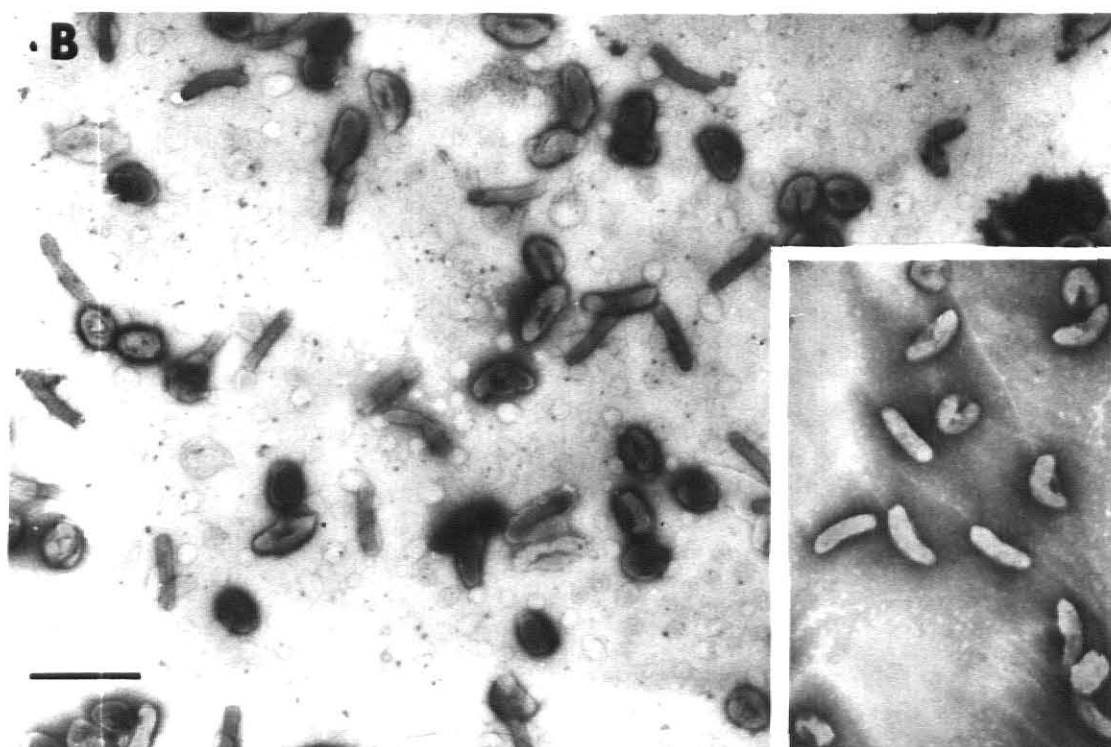
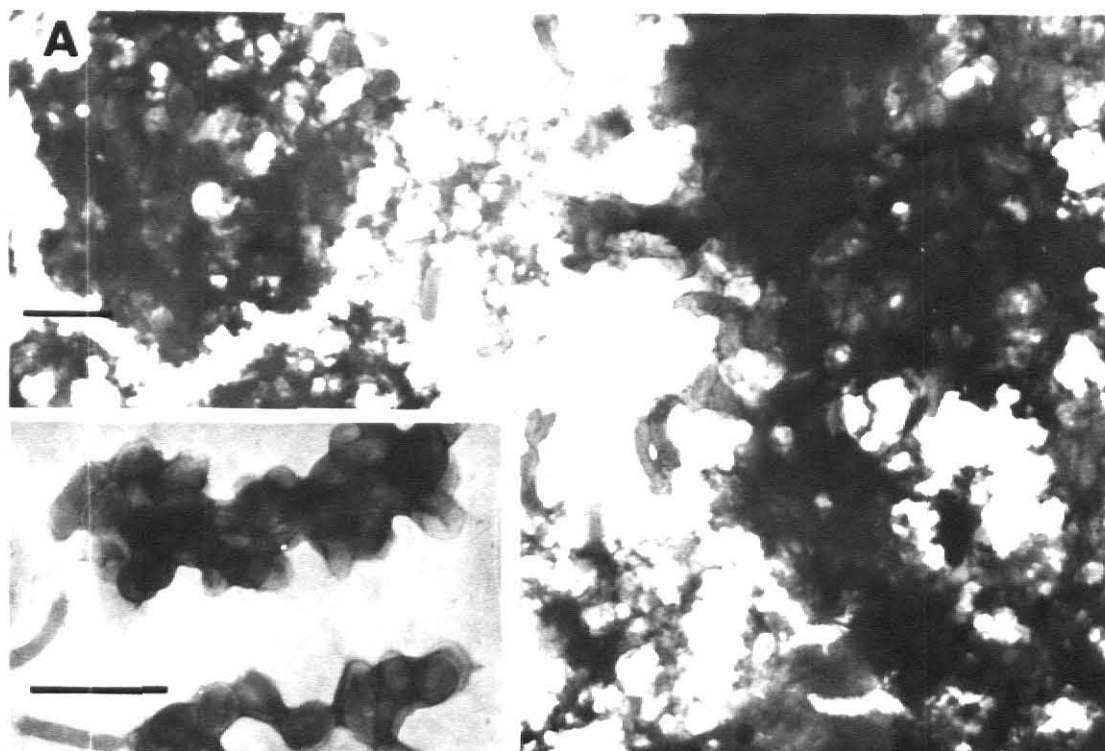
## FIGURE 5.

Electron microscopy of aggregated enveloped nucleocapsids. Samples of aggregated enveloped nucleocapsids at pH 6.6 (A) or enveloped nucleocapsids at pH 7.4 (B) were negatively stained with 2% uranyl acetate. Bars represent a length of 400 nm.

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## SECTION V

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AGGLUTINATION OF VERTEBRATE ERYTHROCYTES BY THE  
GRANULOSIS VIRUS OF Plodia interpunctella

by

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### Abstract

The granulosis virus (GV) of the Indian meal moth, Plodia interpunctella (Hübner) was found to agglutinate several species of vertebrate erythrocytes with differential specificity. Optimal conditions for hemagglutination of rabbit cells were determined to be 0.03 M  $\text{KH}_2\text{PO}_4$ - $\text{Na}_2\text{PO}_4$ , 0.15 M NaCl, pH 6.6 at 25°. The effect of divalent cations on hemagglutination activity was examined;  $\text{Mg}^{2+}$  reduced the titer at concentrations of 0.05 M or higher while  $\text{Ca}^{2+}$  increased the titer at 0.01 M. Electron microscopy and hemagglutination inhibition studies using specific antibody to various viral components indicated aggregated enveloped nucleocapsids were involved in the agglutination phenomenon. Enzymatic treatments of erythrocytes suggested that GV interacted with neuraminidase and trypsin-sensitive cell membrane components. Furthermore, binding studies demonstrated that adsorption of  $^{125}\text{I}$ -GV to cells not only occurred under acidic conditions but also under alkaline conditions where hemagglutination was not observed. It is hypothesized that while GV binds to cells under both acidic and alkaline conditions, hemagglutination is facilitated by aggregation of GV at low pH.