

# Agglutination of Vertebrate Erythrocytes by the Granulosis Virus of *Plodia interpunctella*<sup>1</sup>

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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 <sup>125</sup>I-labeled 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 genus, 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, b, 1978, 1980a, b, c), 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 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,

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chicken, and mouse erythrocytes (Miyajima and Kawase, 1969), and Suto and Kawase (1971) reported a nonoccluded 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, 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 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 Na<sub>2</sub>CO<sub>3</sub>, 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 H<sub>3</sub>BO<sub>3</sub>, 0.098 M NaOH) and mixing at 22° for 10 min. The suspension was then centrifuged at 34,000 in a SW 41 rotor for 120 min at 10°. The pellet containing aggregated enveloped nucleocapsids (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 1 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 twofold dilutions of 0.025 ml of GV-PRH were made in microtiter plates (Microbiological Associates) by using 0.03 M KH<sub>2</sub>PO<sub>4</sub>-Na<sub>2</sub>HPO<sub>4</sub> buffered saline (K-Na<sub>2</sub>PBS) as diluent (Fetjéanu, 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 twofold 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 5 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 legend for Fig. 2. 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 2 weeks and then subcutaneously on the fourth and sixth weeks. Each rabbit received approximately 10 to 50 µg of total protein per injection. Rabbits were bled 5 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<sub>2</sub>HPO<sub>4</sub>, pH 8.0. Preparations of IgG to be used in HAI assays were adjusted to 1 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; and (3) antibody to nucleocapsids would react with nucleocapsid proteins.

HAI assays were performed by making serial twofold dilutions of a 0.025 ml volume of antibody at 1.0 or 0.1 mg/ml IgG ranging from 1:2 through 1:256 in 0.03 M K-Na<sub>2</sub>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 (2 $\times$  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 concentration of 0.75% in PBS, pH 7.2. All treated cells were used immediately. Polyo-ma virus, Newcastle disease virus, and Sendai virus were used as internal controls with enzymatically treated cells.

*Binding assay with rabbit erythrocytes and radiolabeled GV enveloped nucleocapsids.* Gradient-purified enveloped nucleocapsids were surface labeled with  $^{125}$ 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- $\mu$ l aliquot of  $^{125}$ I-labeled EN (specific activity of 42,000 cpm/ $\mu$ g protein) was added to 1.8 ml of a 0.75% suspension of rabbit erythrocytes. The  $^{125}$ I-labeled 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 60 kV.

## RESULTS

### *Species Specificity of Hemagglutination by GV-PRH*

The hemagglutination activity of GV-PRH was found to vary greatly, depending on 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

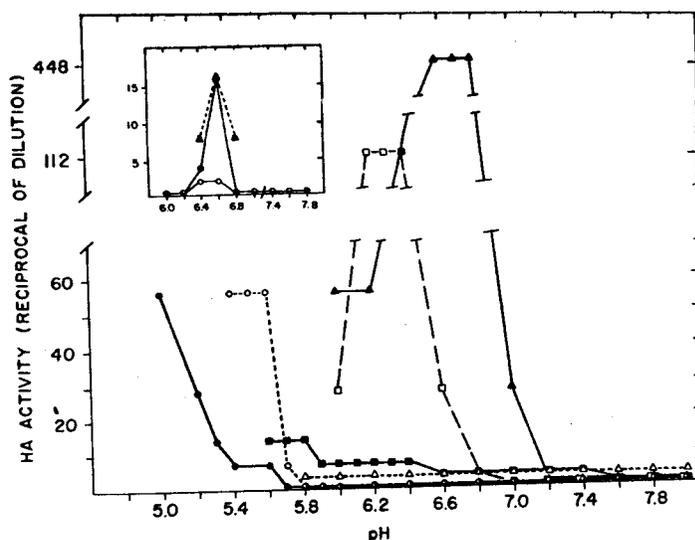


FIG. 1. Effect of pH and erythrocyte species on hemagglutination activity. Serial twofold dilutions of GV-PRH were made in microtiter plates by using K-Na<sub>2</sub>PBS as diluent. The pH varied from 5.0 to 8.0 depending on the species of erythrocyte. Dilutions of GV-PRH ranged from 1:3.5 to 1:7168 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 described under 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.

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 phos-

phate buffer. Control experiments indicated that the PIPES buffer had no detrimental effects on erythrocytes (data not shown). Hemagglutination activity was enhanced twofold 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 to 0.2 M, the activity decreased two- to fourfold. 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

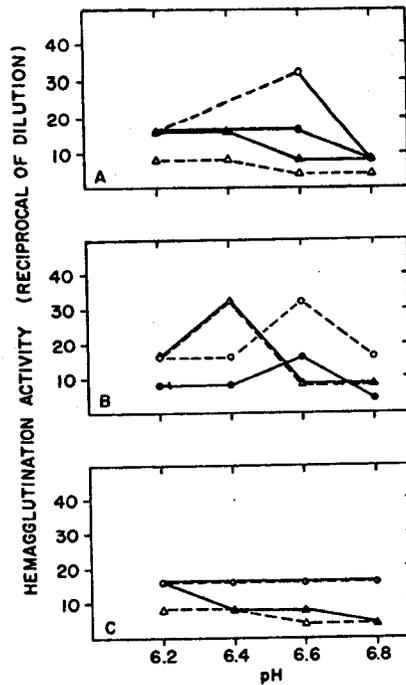


FIG. 2. Optimal conditions for hemagglutination of rabbit erythrocytes: buffer molarity, pH, and temperature. (A) Serial dilutions of GV-PRH were made in the following concentrations of buffer: 0.01 M (●—●), 0.03 M (○—○), 0.6 M (▲—▲), 0.1 M (△—△) K-Na<sub>2</sub>PBS at pH (6.2, 6.4, 6.6, and 6.8), as described under 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°.

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 antioccluded GV and antinucleocapsid IgG, respectively. Similar results were obtained when 8 HA units of GV-PRH were used. In both cases, anti-N or antioccluded GV IgG could inhibit HA only to a small degree as com-

pared with anti-EN IgG. Antigranulin IgG did not inhibit HA at any concentration.

The HAI data suggested that enveloped nucleocapsids were involved in hemagglutination. To substantiate these data further, 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

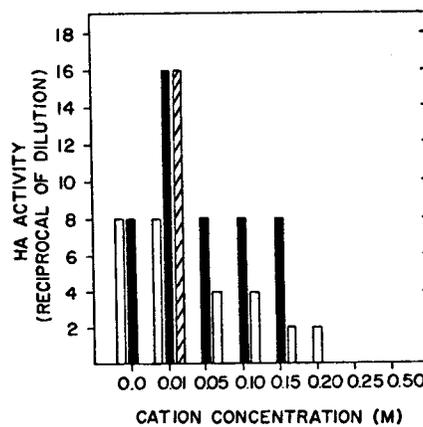


FIG. 3. Effect of divalent cations on hemagglutination of rabbit erythrocytes. Various concentrations of CaCl<sub>2</sub> (■), MgCl<sub>2</sub> (□), and MnCl<sub>2</sub> (▨) were added to 0.02 M PIPES, PH 6.6, which was used as the diluent when serial dilutions of GV-PRH were prepared as described under Materials and Methods. Incubation was at 25° for 120 min. The hemagglutination titer at each cation concentration was determined as described previously.

TABLE 1

## HEMAGGLUTINATION INHIBITION BY SPECIFIC ANTISERA TO GRANULOSIS VIRUS COMPONENTS

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

<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 twofold dilutions of antibody (at 1.0 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.

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 <sup>125</sup>I-Labeled Enveloped Nucleocapsids to Rabbit Erythrocytes*

The binding of purified EN to rabbit erythrocytes was examined by incubating <sup>125</sup>I-labeled 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 cor-

TABLE 2

## EFFECTS OF ENZYMATIC TREATMENT OF RABBIT ERYTHROCYTES ON HEMAGGLUTINATION BY GV-PRH

Viral preparations <sup>a</sup>	Hemagglutination activity			
	Untreated cells <sup>c</sup>	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 nonenveloped 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  $\mu$ g/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°.

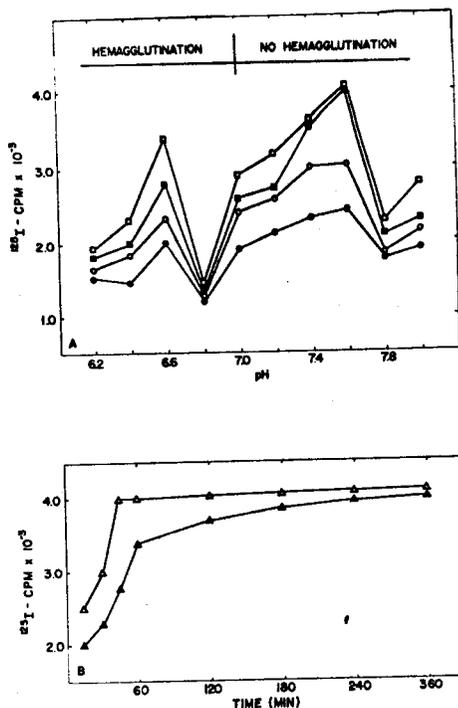


FIG. 4. Binding of  $^{125}\text{I}$ -labeled enveloped nucleocapsids to rabbit erythrocytes. Gradient-purified enveloped nucleocapsids were surface labeled with  $^{125}\text{I}$  and aliquots of labeled virus added to a suspension of erythrocytes in K- $\text{Na}_2$  PBS buffer with pH ranging from 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.  $\bullet$ — $\bullet$ , 15 min;  $\circ$ — $\circ$ , 30 min;  $\blacksquare$ — $\blacksquare$ , 45 min;  $\square$ — $\square$ , 60 min. (B) Total binding observed at pH 6.6,  $\blacktriangle$ — $\blacktriangle$ , and pH 7.6,  $\triangle$ — $\triangle$ , with respect to time.

related 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 6-hr 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  $4^\circ$ . 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 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

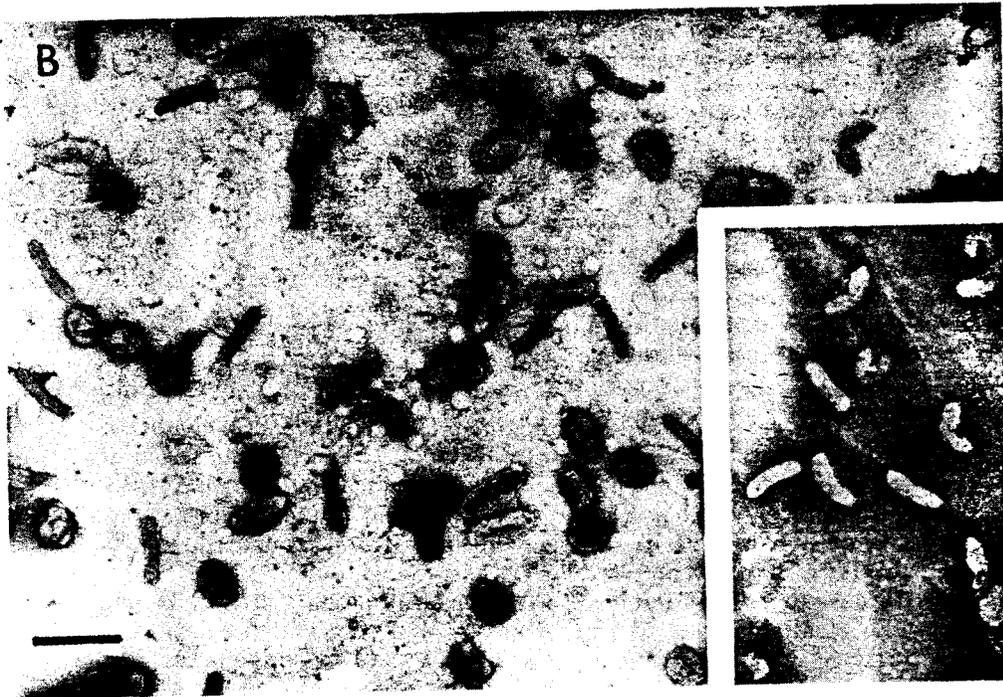
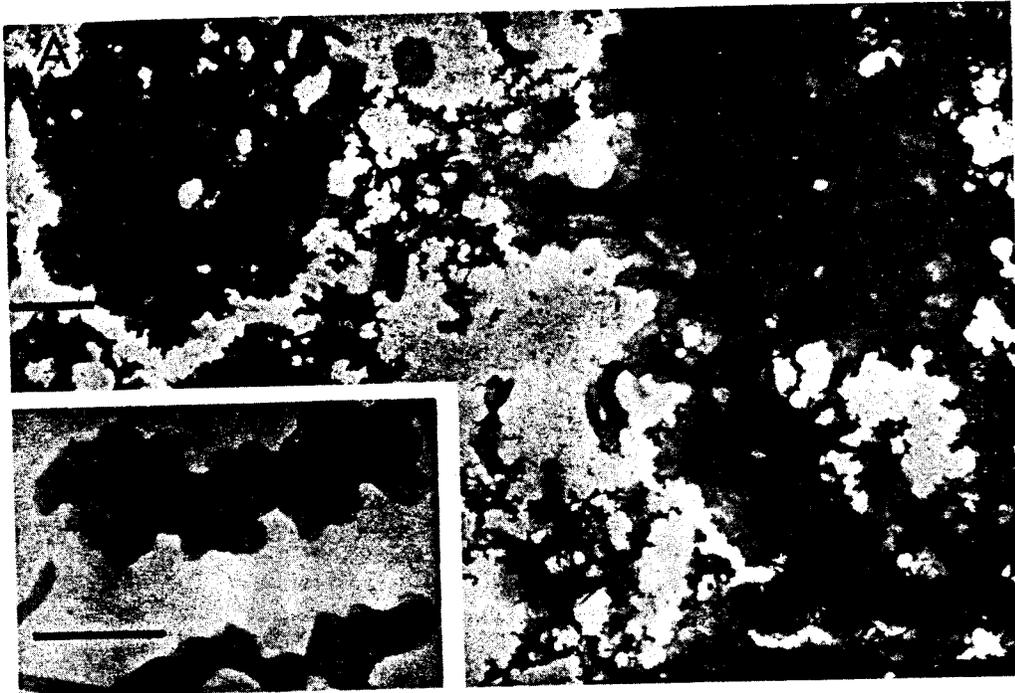


FIG. 5. Electron microscopy of aggregated enveloped nucleocapsids. Samples of aggregated enveloped nucleocapsids at pH 6.6 (A) or enveloped nucleocapsids at pH 7.4 (B) were negatively stained with 2% uranyl acetate. Bars represent a length of 400 nm.

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 infection 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 (Figs. 1 and 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 monova-

lent 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 unprece-

dented, 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 nontarget cells. Such studies are currently underway in our laboratory.

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