Interaction of *Trichoplusia ni* granulosis virus-encoded enhacin with the midgut epithelium and peritrophic membrane of four lepidopteran insects

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Enhacin, an infectivity-enhancing protein from *Trichoplusia ni* granulosis virus (TnGV) was tested for its ability to increase *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus (AcMNPV) infection in the larvae of four lepidopteran insects. Enhacin increased the mortality of AcMNPV infection in all the four insect species tested. Peritrophic membrane (PM) assays showed altered protein profiles in PMs treated with enhacin in all the four species. This supports the hypothesis that enhacin affects virus infection by altering the structural integrity of the PMs. The binding of enhacin to the midgut brush border membranes (BBMs) was determined and specific binding sites were found on the BBM of *Pseudaletia unipuncta*. No specific binding sites were found on the BBMs of *T. ni*, *Helicoverpa zea* or *Spodoptera exigua*. Therefore, specific binding of enhacin to the midgut cell membrane may not be necessary for the enhancement of baculovirus infection in insects.

**Introduction**

Granulosis viruses (GVs) form a genus in the family Baculoviridae (Francki et al., 1991). The GVs from *Pseudaletia unipuncta* (PuGV), *Trichoplusia ni* (TnGV) and *Xestia c-nigrum* have been found to enhance nuclear polyhedrosis virus (NPV) infection in insects (Tanada, 1959; Derksen & Granados, 1988; Goto, 1990). The enhancement of viral infection is due to a protein, encoded by the virus, which is found in the granulin fraction of the viral occlusion bodies (OBs) (Tanada et al., 1973; Hashimoto et al., 1991). The enhancing protein in PuGV was termed synergistic factor (SyF) and that in TnGV was termed viral enhancing factor (VEF). Both of these purified proteins greatly enhance NPV infection in lepidopteran insects (Hukuhara et al., 1987; Gallo et al., 1991).

We recently examined the granulin fraction of 12 GVs by Western blot analysis and detected the presence of cross-reactive proteins in several GVs. It is believed that these enhancing proteins are widespread among GVs and Corsaro et al. (1993) have introduced the name ‘enhacin’ for these virus-encoded proteins. The enhacins from PuGV and TnGV are referred to as En-Pu and En-Tn, respectively.

These baculoviral enhacins were hypothesized to act by degradation of the peritrophic membrane (PM), a barrier against microbial pathogens in insect midguts (Derksen & Granados, 1988), and by binding to insect cell membranes and thereby increasing binding of virions to cells (Tanada, 1985). The hypothesis that enhacins function as binding molecules was based on the finding that En-Pu, when ingested by *P. unipuncta* larvae, bound to insect midgut microvilli (Tanada et al., 1980, 1983; Hara et al., 1976) and the observation that more virions were present on and within the microvilli of insects inoculated with NPV and En-Pu than of those inoculated with NPV alone (Tanada et al., 1975). A more direct observation supporting this hypothesis was that specific binding sites for En-Pu were found on the midgut cell membranes of *P. unipuncta*, and no specific binding was observed on midgut cell membranes from *Bombyx mori*, a species in which NPV infection was not enhanced by En-Pu (Uchima et al., 1988).

In this paper we report the enhancement of infection by *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus (AcMNPV) by En-Tn in the larvae of four lepidopteran insect species, the alteration of protein profiles of the PMs and the binding of En-Tn to midgut brush border membrane (BBM) vesicles. The binding of En-Tn to midgut BBMs was analysed and the results showed that specific binding of En-Tn to cell membranes may not be a requirement for the enhancement of viral infection.

**Methods**

*Insects.* Laboratory colonies of *T. ni* and *Helicoverpa zea* were reared on a high wheatgerm artificial diet (Bell et al., 1981). *Spodoptera exigua* eggs were obtained from the Southern Field Crop Insect Management Laboratory, U.S. Department of Agriculture, Agricultural Research
Table 1. Larval bioassays of enhancement of AcMNPV infection with En-Tn*

(a) Neonate larval bioassay

<table>
<thead>
<tr>
<th>Insect species</th>
<th>Infecting virus dose (OBs/ml)</th>
<th>Treatment†</th>
<th>Insects infected (%)</th>
<th>p</th>
<th>Insects infected (%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. ni</td>
<td>1 x 10^6</td>
<td>Virus + buffer</td>
<td>34</td>
<td>&lt; 0.001</td>
<td>48</td>
<td>&lt; 0.001</td>
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<td></td>
<td></td>
<td>Virus + En-Tn</td>
<td>80</td>
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<td>98</td>
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<td>H. zea</td>
<td>8 x 10^4</td>
<td>Virus + buffer</td>
<td>22</td>
<td>&lt; 0.001</td>
<td>67</td>
<td>&lt; 0.001</td>
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<tr>
<td></td>
<td></td>
<td>Virus + En-Tn</td>
<td>90</td>
<td></td>
<td>96</td>
<td></td>
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<tr>
<td>P. unipuncta</td>
<td>8 x 10^4</td>
<td>Virus + buffer</td>
<td>21</td>
<td>&lt; 0.012</td>
<td>42</td>
<td>&lt; 0.011</td>
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<tr>
<td></td>
<td></td>
<td>Virus + En-Tn</td>
<td>45</td>
<td></td>
<td>68</td>
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<tr>
<td>S. exigua</td>
<td>8 x 10^4</td>
<td>Virus + buffer</td>
<td>6</td>
<td>&lt; 0.001</td>
<td>12</td>
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<td></td>
<td></td>
<td>Virus + En-Tn</td>
<td>84</td>
<td></td>
<td>60</td>
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(b) Fifth instar larval bioassay

<table>
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<tr>
<th>Insect species</th>
<th>Infecting virus dose (OBs/larva)</th>
<th>Treatment‡</th>
<th>Insects infected (%)</th>
<th>p</th>
<th>Insects infected (%)</th>
<th>p</th>
</tr>
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<td>11</td>
<td>&lt; 0.001</td>
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<td></td>
<td>10 x 10^4</td>
<td>Virus + En-Tn</td>
<td>96</td>
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<tr>
<td>H. zea</td>
<td>2 x 10^4</td>
<td>Virus + buffer</td>
<td>9</td>
<td>&lt; 0.005</td>
<td>9</td>
<td>&lt; 0.17</td>
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<tr>
<td></td>
<td>2 x 10^4</td>
<td>Virus + En-Tn</td>
<td>39</td>
<td></td>
<td>19</td>
<td></td>
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<tr>
<td>P. unipuncta</td>
<td>2 x 10^4</td>
<td>Virus + buffer</td>
<td>0</td>
<td>&lt; 0.001</td>
<td>0</td>
<td>&lt; 0.001</td>
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<td></td>
<td>2 x 10^4</td>
<td>Virus + En-Tn</td>
<td>87</td>
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<td>2 x 10^4</td>
<td>Virus + buffer</td>
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<td>&lt; 0.05</td>
<td>5</td>
<td>&lt; 0.05</td>
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<td>4 x 10^4</td>
<td>Virus + En-Tn</td>
<td>33</td>
<td>&lt; 0.05</td>
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</tbody>
</table>

* Forty to 50 insects were used for each treatment.
† En-Tn was added at a concentration of 1 mg/ml throughout (a).
‡ En-Tn was added at a concentration of 1 µg/larva for H. zea, P. unipuncta and S. exigua; for T. ni it was added at 0.2 µg/larva.

Station, Stoneville, Miss., U.S.A. and were reared on high wheatgerm artificial diet. A laboratory colony of P. unipuncta was reared on oats.

Viruses. The AcMNPV used was strain 1A (Wood, 1980) produced in T. ni larvae and purified as previously described (Derksen & Granados, 1988). The TnGV was produced and purified similarly with the following modifications. The TnGV-infected T. ni larvae were homogenized in deionized water (1 ml water per larva) with 10 mM-cysteine to prevent melanization. The homogenate was filtered through four layers of cheesecloth, SDS was added to the filtrate to a final concentration of 1% and the sample was incubated at room temperature for 30 min. Insect debris was removed by centrifugation at 350 g for 7.5 min and the TnGV OBs were pelleted by centrifugation at 11950 x g at 4 °C for 10 min and washed three times with deionized water.

Enhancin preparation. TnGV OBs (1.7 x 10^12 OBs/ml) were dissolved in 0.1 M-Na_2CO_3 for 20 min at room temperature. Undissolved OBs and virions were removed by centrifugation on a 20% sucrose cushion at 160000 x g for 45 min. The supernatant was collected and incubated at 28 °C overnight, the enhancin was purified using a Sephacryl S-300 gel filtration column (Pharmacia; 1.5 cm x 100 cm) and eluted with 0.05 M-Na_2CO_3-NaHCO_3 pH 10.5. The fraction containing En-Tn was collected and concentrated by ultrafiltration with a Centriprep-30 concentrator (Amicon). The En-Tn preparation was quantified using a Bio-Rad Protein Assay kit and stored at -20 °C in water or 0.05 M-Na_2CO_3-NaHCO_3 buffer.

PM preparation. For each insect species, fifth instar larvae were dissected and their PMs were removed. The PMs were washed in water to remove diet residues and then frozen on dry ice and stored at -80 °C (Derksen & Granados, 1988).

Bioassay. Neonate bioassays were conducted as described by Gallo et al. (1991). Briefly, 1 mg/ml of En-Tn was added to each of the AcMNPV inocula and tested for virus infectivity enhancement in four insect species. Fifty neonates were used for each treatment and all bioassays were repeated once with a different batch of En-Tn preparation. The data were subjected to χ² tests to determine the significance of the observed differences.

Fifth instar larval bioassays were performed with T. ni, P. unipuncta, H. zea and S. exigua larvae by following the method for fourth instar bioassay as described by Gallo et al. (1991). Briefly, 1 µl of AcMNPV suspension and 1 µl of buffer (0.05 M-Na_2CO_3-NaHCO_3 pH 10.5) or 1 µl of enhancin were applied to a diet plug in a well of 24-well culture plates. One mid-fifth instar larva was placed into each well and the plates were placed in a 28 °C incubator. When the inoculated diet plugs had been consumed completely, the larvae were individually transferred into 35 ml cups containing artificial diet. A high wheatgerm diet was used for T. ni, H. zea and S. exigua larvae and a pinto bean diet (Quisenberry & Whitford, 1988) was used for P. unipuncta larvae. The cups were then incubated in a 28 °C incubator and the larvae were checked daily. Dead larvae were examined individually under a microscope to confirm that they had died as a result of virus infection. Inoculations with buffer only and En-Tn only served as controls. Each bioassay was repeated once. The significance levels of the data from bioassays were tested by χ² analysis.

In vitro PM assay. One PM was suspended in 40 µl of 0.1 M-Na_2CO_3-NaHCO_3 pH 10.5 containing 5 µg of En-Tn and incubated at 28 °C for 30 min. After incubation, the PM was thoroughly washed in water and then placed in SDS-PAGE sample buffer (Laemmli, 1970) and boiled for 3 min. PMs treated in the same way but without En-Tn served as mock-treated controls. The protein composition of PMs was analysed by SDS-PAGE. Polycrylamide gels (10%) were used for
Trichoplusia ni granulosis virus enhancin

(a) 200K *
(b) 116K -
(c) 66K -
(d) 45K -
(e) 31K --
(f) 22K -
Fig. 1. SDS-PAGE analysis of proteins from the PMs of (a) T. ni, (b) H. zea, (c) P. unipuncta and (d) S. exigua. The asterisks indicate the protein bands that disappeared after treatment with En-Tn and the arrowheads indicate the protein bands that appeared after treatment. The numerals at the left of each gel indicate the location of standard M r markers.

Preparation of BBMs. BBMs were prepared following the procedures of Wolfersberger et al. (1987). Fifth instar larvae were dissected in cold buffer A (300 mM-mannitol, 5 mM-EGTA, 17 mM-Tris-HCl pH 7.5). The midguts were cleared to remove attached tissues, gut contents and PMs, then rinsed thoroughly and placed in liquid nitrogen storage vials. The vials were rapidly frozen in liquid nitrogen and stored at -80 °C. Approximately 2 g of wet midguts was used for BBM preparation. The midguts were thawed and suspended in 18 ml of buffer A at 4 °C, homogenized on ice with a glass homogenizer, mixed with 20 ml of 24 mM-MgCl₂ (ice-cold), homogenized again and left on ice for 15 min. The homogenate was then centrifuged at 2420 g at 4 °C for 15 min. The supernatant was centrifuged at 30590 g at 4 °C for 30 min. The pellet was resuspended in 10 ml of buffer A and mixed with 10 ml of 24 mM-MgCl₂, homogenized and centrifuged at 2420 g for 15 min. The supernatant was centrifuged at 30590 g for 30 min and the BBM pellet was suspended in Grace’s basal medium (Gibco BRL) and stored at -80 °C. The protein concentration of BBMs was determined with a Bio-Rad Protein Assay kit.

Iodination of En-Tn. The En-Tn was iodinated by using IODO-BEADS iodination reagent (Pierce) under the conditions recommended by the manufacturer. The ¹²⁵I (sodium solution) was purchased from Amersham. One IODO-BEADS bead was used with 1 mCi of ¹²⁵I to label 100 µg of En-Tn in about 450 µl of 0.1 M-phosphate buffer pH 7.4. Briefly, one IODO-BEADS bead was washed twice with phosphate buffer, placed in the ¹²⁵I solution (1 mCi in 400 µl of 0.1 M-phosphate buffer pH 7.4), and incubated at room temperature for 5 min. En-Tn (100 µg) was added to the preincubated ¹²⁵I solution and left at room temperature for 15 min. The iodinated En-Tn was separated from free ¹²⁵I with a Bio-Rad Econo-Pac 10DG desalting column (Bio-Rad Laboratories). The fraction of iodinated En-Tn was collected and checked by SDS-PAGE followed by autoradiography. The autoradiogram showed that there was one band in a position identical to that of the unlabelled En-Tn in gels that had been stained with Coomassie blue.

Binding of En-Tn to BBMs. Midgut BBMs (20 to 50 µg protein) were incubated with 10 ng of iodinated En-Tn and 0 to 5 µg of unlabelled En-Tn as competitor in 500 µl of Grace’s basal medium plus 0.5% BSA at room temperature on an orbital shaker for 1 h. The membranes were pelleted by centrifugation at 12000 g for 10 min and carefully washed once, without disturbing the pellet, with 0.5 ml cold Grace’s basal medium containing 0.5% BSA and centrifuged again. The membrane pellet was resuspended in 100 µl of Grace’s medium and transferred to a glass scintillation vial with 5 ml of scintillation cocktail CytoScint ES® (ICN Biomedical). The radioactivity was measured with a Beckman liquid scintillation counter. Reactions with 10 ng of iodinated En-Tn but without BBMs and unlabelled En-Tn were used as blank controls. Scatchard analysis was performed to analyse the binding of En-Tn to BBMs.

Effect of pH on the binding of En-Tn to BBMs. The procedure was similar to that of the binding assay described above except that buffers with different pHs were used instead of Grace’s basal medium. The buffers used for the determination of the pH effect contained 20 mM-citric acid, 20 mM-sodium phosphate and 20 mM-Tris-HCl at pH 5.0, 6.0, 7.0, 8.0 or 9.0. The ionic strengths of the different buffers were kept the same by adding 1 M-NaCl to compensate for the differences in 1 M-NaOH used to adjust for the desired pHs.

Results

Enhancement of viral infection

The neonate bioassays showed that in the presence of 1 mg/ml of En-Tn in the inocula of AcMNPV, mortalities for the four insect species increased significantly
In most instances, mortality was enhanced approximately two- to fourfold in *T. ni, H. zea* and *P. unipuncta*. For *S. exigua*, viral mortalities increased five- to 12-fold. In all cases, $\chi^2$ analysis showed that the differences between controls and enhancin-treated samples were significant below the 5% level of probability.

The fifth instar larval bioassays also showed that by addition of 0.2 or 1 $\mu$g of En-Tn to the inoculum diet plug, virus infection was significantly enhanced (Table 1b). For *T. ni* larvae, the infection rate was increased from less than 20% to more than 90%. For *P. unipuncta*, no infection occurred in the absence of En-Tn, whereas 82 to 87% of larvae were infected in the presence of En-Tn. En-Tn increased the percentage of infected *H. zea* and *S. exigua* larvae, but not to the same extent observed with *T. ni* or *P. unipuncta*. Helicoverpa zea does not support high levels of AcMNPV replication (Vail & Vail, 1987) and this may explain the low significance level in experiment 2 (Table 1b). No virus infections were observed in the buffer only controls or in En-Tn only controls.

The death of fifth instar *T. ni, P. unipuncta* and *H. zea* always occurred in the larval stage. In the case of *S. exigua*, mortality was observed in both the larval and pupal stages. However, only the larval mortality is reported from these bioassays (Table 1b).

**SDS-PAGE of PM proteins**

The *in vitro* PM assays showed that En-Tn altered the protein profiles from the PMs of all four insect species tested (Fig. 1). SDS-PAGE of En-Tn-treated PMs from *T. ni* showed a protein profile similar to that reported by Derksen & Granados (1988). Some high $M_r$ proteins disappeared and some lower $M_r$ protein bands appeared (Fig. 1a). A similar pattern of disappearance of high $M_r$ proteins and the appearance of low $M_r$ proteins was seen in the PMs of *H. zea*, *P. unipuncta* and *S. exigua* treated with En-Tn. The most significant changes in PM protein profile were observed with *T. ni*.

**Binding of En-Tn to BBMs**

Competition for binding between unlabelled and labelled En-Tn was demonstrated in *P. unipuncta* BBMs but not in BBMs of the other three species tested (Fig. 2). When BBMs (20 $\mu$g protein) from *P. unipuncta* were incubated with 10 ng of iodinated En-Tn in 0.5 ml of Grace’s medium, in the presence of 500-fold excess unlabelled En-Tn the binding decreased from approximately 24% (without unlabelled competitor) to 10% (Fig. 2a). The binding of iodinated En-Tn to the BBMs from the other three insect species with excess unlabelled En-Tn was unchanged from that without unlabelled En-Tn (Fig. 2b to d). This demonstrated no specific binding sites on the midgut BBMs of these three insect species (*T. ni, S. exigua* and *H. zea*), although low levels of non-specific binding occurred on the BBMs of all the four insect species tested.

**Scatchard analysis of the binding of En-Tn to BBMs**

The binding of En-Tn to BBMs from *P. unipuncta* and *T. ni* was further tested by adding several concentrations of unlabelled En-Tn to compete with the binding of iodinated En-Tn to the BBMs. Binding of the iodinated En-Tn to the BBMs of *T. ni* was not affected in the presence of 0 to 5 $\mu$g of unlabelled En-Tn (Fig. 3a). In
Trichoplusia ni granulosis virus enhancer

Fig. 3. Competitive binding assays for $^{125}$I-En-Tn binding to the BBMs of (a) T. ni and (b) P. unipuncta. Increasing concentrations of unlabelled En-Tn were mixed with 10 ng of labelled En-Tn.

Fig. 4. Scatchard plot of the binding of En-Tn to the BBMs from P. unipuncta from data shown in Fig. 3. The solid line represents observed data and the dotted line represents specific binding.

Contrast the binding of iodinated En-Tn to the midgut BBMs from P. unipuncta decreased with the addition of increasing amounts of unlabelled En-Tn, demonstrating competition for sites on the BBMs (Fig. 3b). The Scatchard analysis of these data showed that En-Tn bound specifically to the BBMs from P. unipuncta with a dissociation constant of $1.9 \times 10^{-8}$ M. There were $3.2 \times 10^{-12}$ mol of binding sites on 20 $\mu$g of BBM proteins from P. unipuncta (Fig. 4).

**Effect of pH on the binding of En-Tn to BBMs**

The effect of pH on the binding of En-Tn to BBMs from P. unipuncta and T. ni was determined (Fig. 5). The binding of En-Tn to T. ni BBMs did not show any specificity from pH 5-0 to 9-0 (no competitive effect), although the binding increased with an increase in acidity (Fig. 5a). The binding of En-Tn to BBMs from P. unipuncta showed that the total as well as non-specific binding increased with an increase in acidity. The difference between total and non-specific binding represents specific binding. From pH 5-0 to 6-0 the specific binding was the highest. Specific binding decreased as the pH increased from 6-0 to 8.0. At pH 8-0 to 9-0 specific binding was near zero (Fig. 5b).

**Discussion**

En-Tn enhances AcMNPV infection in neonate and late instar T. ni larvae (Derksen & Granados, 1988; Gallo et al., 1991). The larval bioassays reported in this study clearly showed that En-Tn enhanced AcMNPV infection in H. zea, S. exigua and P. unipuncta in addition to T. ni (Table 1). This finding suggests that En-Tn may enhance virus infection in a broad range of species in the family Noctuidae (Lepidoptera). One microgram of En-Tn per fifth instar larva resulted in enhancement of AcMNPV infection. The lowest concentration of En-Tn needed for virus enhancement was not determined in this study.

Derksen & Granados (1988) showed that En-Tn disrupted the integrity of the PM of T. ni larvae. By SDS-PAGE analysis, they demonstrated that in TnGV-treated T. ni PM, high $M_r$ proteins disappeared and low $M_r$ protein bands appeared. Our results demonstrated the disappearance of T. ni PM proteins after En-Tn treatment and concur with the observations of Derksen & Granados (1988) (Fig. 1). In addition, the in vitro PM assays showed that En-Tn treatment resulted in an altered PM protein profile in the PMs of the other three...
species *H. zea*, *P. unipuncta* and *S. exigua*. The altered PM protein profiles are different from species to species. In general, high *M*<sub>r</sub> proteins disappeared and low *M*<sub>r</sub> proteins appeared after treatment. The PM assays showing the altered protein profile of the PMs were correlated with larval bioassays which showed infection enhancement in the fifth instar insect larvae of all species tested (Table 1b). Neonate *in vitro* PM assays are not feasible because of the extremely small size of the animals; however it has been reported that neonate PMs may be structurally similar to those found in fifth instar larvae (Ryser et al., 1992). The disappearance of PM proteins from all four insect species that were treated with En-Tn further strengthens the hypothesis that En-Tn enhances virus infection in larvae by altering the integrity of the PM (Granados & Corsaro, 1990; Corsaro et al., 1993).

Uchima et al. (1988) found specific binding sites for En-Pu on the midgut cell membranes of *P. unipuncta* larvae but no specific binding sites on the midgut cell membranes of *B. mori*. The presence and absence of the specific binding sites on the midgut membranes of these two insects was correlated with the function of En-Pu. Uchima et al. (1988) suggested that the En-Pu acted as a binding molecule which enhanced virus infection as proposed by others (Tanada, 1985). We found that there are specific binding sites for the En-Tn on the midgut BBMs of *P. unipuncta* but not on those of three other insect species (Fig. 2). However En-Tn enhances virus infection in all four insect species. Therefore, specific binding sites may not be a prerequisite for enhancement of virus infection in insects.

Scatchard analysis (Fig. 4) showed that En-Tn binds to the BBMs of *P. unipuncta* with a dissociation constant of 1.9 × 10<sup>-8</sup> M and that there were 3.2 × 10<sup>-12</sup> mol of binding sites per 20 µg membrane protein. The dissociation constant for the En-Pu reported by Uchima et al. (1988) is 1.57 × 10<sup>-9</sup> M and they measured 4.20 × 10<sup>-13</sup> mol of binding sites per 50 µg membrane protein. The Scatchard plot of En-Tn binding to BBMs of *P. unipuncta* showed a flat slope following the initial steep slope (when [Bound] is above 4 nM). We consider this flat slope to be representative of non-specific binding to BBMs.

The binding, either specific or non-specific, of En-Tn on midgut BBMs at pH 5.0 to 6.0 was the highest and binding at pH 6.0 to 9.0 was near zero. Uchima et al. (1988) reported that the optimal pH for specific binding of En-Pu to midgut cell membranes was pH 6.0. We did not observe a decrease in specific binding at pH 5.0. Specific binding of En-Tn on BBMs of *P. unipuncta* was high at pH 5.0 to 6.0, decreased at pH 6.0 to 8.0 and approached zero at pH 8.0 to 9.0. The environment surrounding the brush border in the midgut of live insects is approximately pH 7.5 to 7.8 (Santos et al., 1983; Yunovitz et al., 1987). Therefore, the pH around the midgut microvilli, the site for virus attachment and entry, does not appear to be optimal for specific binding of either En-Pu or En-Tn.

This study showed that the En-Tn altered the protein profile in the PMs. This supports the hypothesis that viral enhancers increase virus infection by altering the structural integrity of the midgut PMs in insects (Derksen & Granados, 1988). The function of specific binding of En-Tn and En-Pu to *P. unipuncta* midgut membranes is not fully understood at this time. However, we cannot rule out the possibility that these enhancers may somehow mediate the attachment and entry of virus particles into midgut cells.

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**References**


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