In Vitro Infectivity of the Autographa californica Nuclear Polyhedrosis Virus Under Various Conditions

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SUMMARY

The stability of the in vitro infectivity of the non-occluded virions (NOV) of the Autographa californica nuclear polyhedrosis virus (Ac-NPV) was tested. Exposure of Ac-NPV NOV to solutions ranging from pH 7.5 to 11.4 with molarities of 0.1 to 0.001 showed that infectivity was most stable in 0.001 M, pH 7.5, tris(hydroxymethyl)aminomethane (tris)-HCl buffer. Infectious NOV could be concentrated by precipitation with 50% saturated ammonium sulphate or 9% (w/w) polyethylene glycol 4000. Treatment of concentrated preparations with 1 M-urea did not affect infectivity or the centrifugal properties of the NOV. NOV were partially purified by centrifugation through a sucrose underlay.

Replication of the Autographa californica nuclear polyhedrosis virus (Ac-NPV) in insect tissue culture cells results in the production of both occluded (OV) and non-occluded (NOV) forms of the virus. Following alkali release of OV from the inclusion bodies, the OV is 0.06% as infectious in vitro as the NOV on a per particle basis, and the two forms differ serologically (Volkman et al. 1976). The OV and NOV particles are therefore considered to be biologically different forms of the virus. To study the reason for the greater in vitro infectivity of NOV, investigations were conducted to determine the best conditions for stabilization of NOV infectivity during purification.

The infectivity of Ac-NPV NOV has been shown to be sensitive to organic solvents and acid (Dougherty et al. 1975). Similarly the NOV of Trichoplusia ni NPV are sensitive to detergents (Henderson et al. 1974). Both results indicate that the membrane enveloping these particles is required for infection.

With the development of plaque assay procedures (Hink & Vail, 1973; Brown & Faulkner, 1977; Wood, 1977), the stability of NOV infectivity under a wide range of conditions can now be assessed critically.

The ovarian cell line of Trichoplusia ni (TN-368) grown in TNMFH medium (Hink, 1970) at 26 °C was used in this study. The Autographa californica nuclear polyhedrosis virus (Ac-NPV) was a gift from Dr P. V. Vail (Phoenix-Western Cotton Research Laboratory, Phoenix). During this study the virus was transferred for four passages in tissue culture. After four passages, third-instar larvae of Estigmene acrea were fed 10^6 inclusion bodies per insect. Seven days post-inoculation, infectious haemolymph was obtained from cut prolegs and frozen at −70 °C. The haemolymph was judged to contain only Ac-NPV on the basis of polyacrylamide gel electrophoresis of virus proteins (H. A. Wood, unpublished data), and was used to produce first passage infectious supernatant (IS) which contained the NOV. IS was obtained by inoculation of 7.5 × 10^6 logarithmic phase cells with 3.75 × 10^7 plaque forming units in a total volume of 15 ml of growth medium. At 36 to 44 h post-inoculation, the cells were pelleted by centrifugation at 800 g for 5 min and the IS filtered through a 0.45 μm Millipore filter. The infectivity of all IS preparations was determined by a plaque assay (Wood, 1977) and they were stored at 4 °C.
Titrated IS samples were diluted 10^{-5} and 10^{-6} in 0.1 to 0.001 M-borate, tris and phosphate buffers and carbonate solutions with ranges of pH from 7.5 to 11.4. Control samples were diluted in TNMFH medium. All samples were stored at 4 °C for 7 to 10 days and then infectivity was assayed according to Wood (1977). Eight replications of each dilution were assayed, and all samples were assayed using a common cell suspension. Infectivities were expressed as a percentage of control infectivity.

Virus samples were also pelleted at 41,000 g for 1 h at 4 °C using a Beckman Ti 50 rotor and a 1 ml underlay per tube of 30 % (w/w) sucrose in each of the diluents mentioned above. The pellets were resuspended and subjected to dialysis for 7 days in their respective diluents. Samples were then adjusted to equal volumes, diluted in TNMFH medium and assayed.

The IS samples were centrifuged in a DuPont Sorvall OTD-2 ultracentrifuge equipped with an automatic rate controller, or in a Beckman L2-65-B ultracentrifuge. Sterile polycarbonate tubes were used, and the samples were centrifuged at 40,000 g for 45 or 90 min at 4 °C in a Beckman Ti 50 rotor. In some tubes a 0.5 ml underlay of 30 % (w/w) sucrose in 0.001 M-tris-HCl buffer, pH 7.5, was used. The infectivity of pellet and supernatant fractions was assayed.

Polyethylene glycol 4000 (PEG) and NaCl solutions at varying concentrations were added to IS samples. These were intermittently mixed in an ice bath for 2 h. The samples were then centrifuged at 1000 g for 20 min. The pellets were resuspended in 0.001 M-tris-HCl buffer, pH 7.5. The supernatant and pellet fractions were diluted in TNMFH medium and infectivity assayed.

Varying amounts of a 50 % saturated solution of (NH₄)₂SO₄ were added to IS and the mixtures held in an ice bath with intermittent stirring for 60 min. The samples were centrifuged for 20 min at 1000 g. Following precipitation of the virus, the infectivities of the supernatant and pellet fractions were assayed. Pellets were resuspended in 0.001 M-
tris-HCl buffer, pH 7.5, containing 0.5 or 1.0 M-urea. Samples were incubated at room temperature for 30 min, then diluted and infectivity assayed.

Density gradients were prepared by layering 1.5, 1.5, 1.5, 1.5, 1.5 and 2.5 ml of 25, 30, 35, 40, 45, 50 and 55 % (w/w) sucrose solutions, respectively, in 0.001 M-tris-HCl buffer, pH 7.5. The columns were allowed to diffuse for 24 h at 4 °C. Samples of 1 to 0.5 ml were layered on to the gradients and centrifuged for 5 h at 150,000 g in a Beckman SW 41 rotor at 4 °C. The gradients were fractionated with an Isco density gradient fractionator (Isco, Lincoln, Nebraska). Infectivity of single drop fractions taken at 0.5 ml intervals throughout the gradient was assayed. The density of the remaining fractions was determined by refractometry.

The titratable infectivity of the NOV in IS of Ac-NPV was strongly influenced by the concentration and pH of the solutions tested. The data in Table 1 are averages for three dilution:storage experiments. Statistical analysis of the data was performed using a factorial analysis of variance and Tukey's test for comparison of means. The statistical analysis showed that the 0.001 M-tris-HCl buffer, pH 7.5, was the only buffer which consistently ranked in the upper set at the 10 % significance level of differences. With each diluent, the observed residual infectivities at each molarity were significantly different at the 10 % level.

Experiments in which the NOV was pelleted by centrifugation and dialysed against the buffers gave comparable results to those reported in Table 1 but greater variability occurred between experiments.

Additional experiments were conducted to test infectivity following dilution and storage in pH 7.5 tris-HCl buffers at 0.1, 0.05, 0.01, 0.005 and 0.001 M as well as in glass-distilled water. The average residual infectivities from three experiments after one week of storage were 35, 37, 43, 46, 96 and 26 % of the medium control, respectively. Preliminary experiments show that IS incubated in pH 7 and 6.5 phosphate buffers is less infective than that incubated in phosphate buffer at pH 7.5.

A short term experiment was performed in which NOV was treated with 0.05 M-sodium carbonate for up to 1 h prior to assaying. There was no observed loss in infectivity.

Neither aggregation of the particles nor buffer effects upon the cell:virus interaction are considered to be the major factors contributing to the results in Table 1. Firstly, the virus preparations were in a highly diluted state during exposure to these conditions. Secondly, the NOV treated for 1 h in 0.05 M-sodium carbonate did not have a decreased infectivity, indicating that high pH and salt concentration had no significant effect on the monodispersity of the particles or cell:virus interactions. In addition, the dialysis treatments in which the NOV at higher concentrations were exposed to these conditions gave similar results. The greater variability observed in these experiments is attributed to the multi-step procedure used.

Similar ionic strength effects were obtained by Kawanishi & Paschke (1970). Their data indicated that virions of Rachiplusia ou NPV, when liberated from inclusion bodies, lost structural integrity and infectivity at high ionic strengths. Harrap & Longworth (1974) recommended the use of distilled water to maintain the structural integrity of granulosis virus particles released from inclusion bodies. With Ac-NPV NOV, although low ionic strength conditions are desirable, storage in distilled water is deleterious.

Concentration of NOV by centrifugation resulted in more than 90 % recovery of infectivity in the pellets with or without the sucrose underlay, following centrifugation for 45 min. After 90 min centrifugation there was a 10 to 20 % decrease in the observed titres of samples pelleted without the underlay. This loss may have arisen due to slight aggregation.
Table 2. Polyethylene glycol 4000 and ammonium sulphate precipitation of non-occluded Autographa californica NPV

<table>
<thead>
<tr>
<th>% PEG (w/v)*</th>
<th>Precipitated p.f.u.†</th>
<th>Total p.f.u. in sample‡</th>
</tr>
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<tbody>
<tr>
<td>12</td>
<td>0.95</td>
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</tr>
<tr>
<td>9</td>
<td>0.95</td>
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</tr>
<tr>
<td>6</td>
<td>0.18</td>
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<tr>
<td>3</td>
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<tr>
<td>0</td>
<td>0.02</td>
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<tr>
<td>% saturation with (NH₄)₂SO₄</td>
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<tr>
<td>80</td>
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<td>75</td>
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<td>66</td>
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<td>50</td>
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<td>33</td>
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<tr>
<td>20</td>
<td>0.18</td>
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<tr>
<td>10</td>
<td>0.09</td>
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</table>

* All mixtures contained 0.2 M-NaCl.
† Plaque forming units determined according to Wood (1977) using eight replications per treatment.
‡ Titre of untreated samples.

In all cases the supernatant fractions contained less than 1% of the total infectivity of the sample. Pelleting through the sucrose underlay resulted in virus pellets which were smaller and more opalescent.

Concentration of NOV from IS was obtained by PEG precipitation at about pH 6.8 (Table 2). At 9 to 12% (w/v) PEG and 0.2 M-NaCl, 95% of the virus infectivity was recovered in the pellets. Addition of 12% (w/v) PEG without NaCl resulted in precipitation of 79% of the virus infectivity.

NOV could also be concentrated by precipitation with ammonium sulphate (Table 2). Maximum precipitation of infective particles occurred at 50 to 66% saturation; at higher concentrations there was a decrease in total recoverable infectivity. The complete recovery of the infectivity of samples precipitated in 50% saturated ammonium sulphate also indicates that the virions can tolerate relatively high salt conditions for short periods of time.

Both the ammonium sulphate and PEG precipitation of NOV were useful as fast and efficient means of concentrating NOV from IS. However, differential precipitation was not useful for purification because most of the other proteins in the IS precipitated under the same conditions as NOV. Therefore, precipitation under these conditions may occur due to physical co-precipitation with other proteins rather than it being a specific property of the virus.

As a means of dissociating contaminating host proteins from NOV during concentration, ammonium sulphate precipitated samples were resuspended in 0.001 M-tris-HCl buffer, pH 7.5, containing 0.5 or 1.0 M-urea without loss of infectivity. Treatment with 1 M-urea also greatly reduced the amount of pelleted material in the NOV preparations. When urea-treated and control samples were subjected to quasi-equilibrium sucrose density gradient centrifugation for 5 h, no difference in the distribution of infectivity was detected. The major peak of infectivity occurred at approx. 38% (w/w) sucrose and a minor peak at 48% (w/w) sucrose. Centrifugation for 24 h gave similar results but the peaks were broader due to a change in the gradient profile. Since the occurrence of the minor peak was variable and the
conditions were quasi-equilibrium, it is considered that the minor peak may have arisen
due to aggregation between particles and/or between particles and cellular debris.

In summary, the conditions tested herein indicate that the NOV form of Ac-NPV can
tolerate a wide range of purification procedures while retaining its infectivity in vitro.
Experimentation is now being conducted to evaluate further and improve the conditions
necessary to obtain highly purified and infectious NOV preparations.

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Boyce Thompson Institute
Yonkers, New York 10701, U.S.A.

H. A. WOOD
G. SPEYER

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