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Replication of a Nuclear Polyhedrosis Virus in a Continuous Cell Culture of *Spodoptera frugiperda*: Purification, Assay of Infectivity, and Growth Characteristics of the Virus

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Nonoccluded virus, polyhedra, and occluded virus were purified from a continuous cell culture of *Spodopera frugiperda* infected with nuclear polyhedrosis virus. The optimal temperature for the replication and lateral transmission of infectivity for the nuclear polyhedrosis viruses (NPV) in cell culture was 27 C. End-point dilution and plaque assay procedures for the measurement of infectivity are described and compared. Dose-response data demonstrated that a single particle could initiate an infection, and the validity of the relationship of 0.7 PFU per mean tissue culture infective dose ($TCID_{50}$) further substantiated the accuracy of these infectivity assays. Particle-infectious unit calculations gave a ratio of 62 to 310 nonoccluded virus particles $TCID_{50}$. Growth cycle and lateral transmission experiments indicated that infectious material was released from cells 12 h postinfection (p.i.) and approached a maximal titer 4 days p.i. The number of polyhedra, nonoccluded virions, and $TCID_{50}$ produced per cell was also presented. Typical yields of NPV produced per liter flask suggested that insect cell culture systems represent a feasible means by which the replication of these viruses could be investigated.

Nuclear polyhedrosis viruses (NPV) are members of the *Baculovirus* genus (18) and have been isolated from insects in the orders Diptera, Hymenoptera, Lepidoptera, and Orthoptera (6). The virion is bacilliform (40 to 70 by 250 to 400 nm) and contains covalently closed, supercoiled, double-stranded DNA with a molecular weight of approximately 10^8 (13-15). The virus replicates in the nucleus of the insect cell, and, as the infection proceeds, a substantial proportion of the progeny are enveloped and subsequently occluded by protein into polyhedral, crystalline matrixes (0.1 to 10 μm in diameter). The inclusions or polyhedra are readily detectable in the nuclei of infected insect cells and present a cytopathology that is characteristic of infections initiated by viruses of this genus (3-5).

Polyhedra represent the primary "vector" by which virus infections are transmitted in nature to insects. When larvae ingest foliage that is contaminated with polyhedra, the alkaline environment and enzymatic activity associated with the larval gut solubilized the protein matrix of the polyhedron, releasing infectious virions (6). Polyhedra are not infectious for insect cell cultures (11), but cultures do support the replication of these viruses when alternative sources of inoculum are used. For example, cultures have been infected with hemolymph of NPV-infected insects (1, 2, 12), with purified NPV DNA (11), with NPV-infected cell culture extracts (1, 11, 16), and with cell-free extracts of NPV-infected larvae (16). There is, however, only one report where the virus was purified from infected cell cultures and shown to be infectious (8).

Infectivity assays of polyhedra have relied primarily on a mean lethal dose fed to larvae. An end-point dilution method using a primary insect cell culture (17) and a quantal response method using a continuous cell culture (1) have also been used to assay infectious material other than polyhedra. More recently, the first plaque method using 0.6% methylcellulose as an overlay has been reported (10).

In this paper data concerning aspects of the replication of the NPV of *Spodoptera frugiperda* in a continuous cell culture derived from *S. frugiperda* is presented. Purification procedures are described, and the temperature optimum for the replication of the virus in the cell culture is established. Infectivity assay procedures for an end-point dilution method and a plaque method are also described and compared with particle count data, and the particle-infectious

unit ratio is calculated. The growth characteristics of the virus in cell culture are also investigated.

MATERIALS AND METHODS

Cells. The continuous cell culture was originally established from pupal ovaries of *S. frugiperda*, the fall armyworm (J. L. Vaughn, unpublished data), and it has been adapted to a modified medium, BML/TC10 (TC100) (G. R. Gardiner and H. Stockdale, Proc. VIth Annu. Meet. Soc. Invertebr. Pathol., Oxford, England, Abstr., 1973). The cells were received at the 57th passage level from H. Stockdale and have been passaged over 50 times in this laboratory. The cells were polyploid, grew attached to the surface of the culture vessel, and proliferated with a population doubling time of approximately 24 h. The cells were routinely subcultured at weekly intervals by removing them from the surface of the culture vessel using a rubber scraper and vigorously pipetting until a uniform suspension was obtained. Viable cell counts were made by using trypan blue and a hemocytometer. Glass flasks (32 oz. [ca. 7 g]) were seeded with 10^6 cells and incubated at 27 C (the optimal temperature for growth of the cells) until confluency was reached (approximately 2.5×10^7 cells).

TC100 was prepared complete, sterilized by positive pressure filtration through 142-mm membrane filters (Millipore Corp.), and stored at 4 C. Before it was used, penicillin-streptomycin (100 to 200 U/ml) and kanamycin (100 to 200 μg/ml) were added.

Virus. The virus was received from two sources: one from H. Stockdale as infected cell cultures and the other from J. L. Vaughn as polyhedra of *S. frugiperda* NPV. The infected cell cultures were initiated by Dr. Vaughn, and the viruses, therefore, were considered equivalent, even though they were received from different sources. Further, no differences have been detected in this laboratory that would suggest the contrary. The NPV was the multiply embedded type, i.e., virions are occluded by the polyhedral protein matrix as groups or bundles with more than one virion per group. Since it is well established that serial passage of virus in cell cultures frequently results in aberrant virus forms, the NPV produced in cell culture was always kept within 10 passages of a passage through the insect. The methods for the production and purification of NPV from insects have been described elsewhere (K. A. Harrap and J. F. Longworth, J. Invertebr. Pathol., in press).

The NPV was routinely produced in liter flasks which were seeded with 2.5×10^6 cells, incubated for 24 h, and infected during the logarithmic growth phase of the cells at a multiplicity of infection (MOI) of 0.01 mean tissue culture infective dose ($TCID_{50}$)/cell. The uninfected cells grew under these conditions and formed a confluent monolayer. They were harvested after 7 to 10 days of incubation, when the monolayer degenerated and a proportion of the cells had detached from the surface of the culture vessel.

Incorporation of radioactive precursors. Radioactive NPV was produced by seeding 75-cm^2 Falcon Flasks with 2.5×10^6 cells/10 ml, incubating for 24 h, and infecting with an MOI of 0.1 $TCID_{50}$ per cell. Twenty-four hours later, 10 μCi of [*methyl*-^{3}H]thymidine per ml (15,000 to 30,000 mCi/mmol; Radiochemical Centre, Amersham) was added to each flask, which was incubated for 7 to 10 days before the virus was harvested. All radiochemical data presented in this paper are expressed as trichloroacetic acid precipitable counts/min. The labeled samples were prepared for counting by adding a carrier, bovine serum albumin fraction V, to give a final concentration of 50 μg/sample when diluted with an equal volume of 15% (wt/vol) trichloroacetic acid. The samples were left at 4 C for at least 1 h, and the precipitates were collected by suction filtration through 25-mm glass fiber disks (GF81, Whatman) and washed with 10 ml of cold 7.5% trichloroacetic acid and ethanol. The disks were dried and placed into glass vials, and 10 ml of scintillant was added (4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-di[2-(5-phenyloxazolyl)]-benzene per liter of toluene). The samples were counted in a Phillips liquid scintillation analyzer PW4510/00 with a 40% counting efficiency for ^{3}H.

Purification. Infected cultures were harvested, and the suspension was pelleted at $10,000 \times g$ for 20 min, removing cellular debris and polyhedra and leaving virus that had not been incorporated into polyhedra (nonoccluded virus) in the supernatant. The 10,000 *g* pellet was resuspended in a small volume of water and sonicated at 4 C for 30 s at 5 A using a Dawe Soniprobe type 1130A fitted with a soniprobe converter type 1130/1A. The sample was repelleted, and the 10,000 *g* supernatants were pooled. This pellet represented the crude polyhedra fraction. The 10,000 *g* supernatant was pelleted at $30,000 \times g$ for 1 h, and the supernatant was discarded. The 30,000 *g* pellet represented the crude nonoccluded virus fraction.

The 10,000 and 30,000 *g* pellets were resuspended in water and layered onto 15-ml linear gradients of 1.0 to 2.25 M sucrose in water. The gradients were centrifuged at $60,000 \times g$ for 1.5 h, and the visible polyhedra and virus bands were collected and repelleted, respectively. The polyhedra were recentrifuged on gradients to obtain a fraction of polyhedra devoid of cellular debris. The gradient-purified nonoccluded virus was resuspended and pelleted three times at $2,000 \times g$ for 15 min aseptically, and the supernatant was stored frozen at −20 C. The polyhedra were either stored frozen or subjected to an alkali dissolution procedure (Harrap and Longworth, in press) to yield gradient-purified occluded virus.

The gradients were centrifuged at 4 C and fractionated by displacement with constant volumes of liquid paraffin. The percent (wt/wt) sucrose was determined by using an Abbé "60" refractometer that was attached to a circulating Haake FE constant-temperature water bath set at 25 C.

Temperature optimum. Leighton tubes (16 by 120 mm) containing 9 by 35 mm glass cover slips were seeded with 5×10^5 cells/ml, and the virus inoculum (0.2 ml) was adjusted to yield approximately 20% of the cells infected after 24 h of incubation at 27 C. The cultures were inoculated, incubated at the appropriate temperature (±1 C), and scored daily for 6 days

for the percentage of cells exhibiting a cytopathic effect (CPE).

End-point dilution method for the assay of infectivity. Leighton tubes were seeded with 5×10^4 cells/ml and incubated for 24 h. The virus to be assayed was serially diluted in TC100, and 0.2 ml of each dilution was added per tube using five tubes per dilution. Where necessary the medium was decanted before inoculation and, once inoculated, the tubes were left at ambient temperature for 1 h. They were washed three times with 1 ml of TC100 and a final milliliter of medium was added. The cultures were incubated for 5 to 7 days, or until confluency was reached, and prepared for scoring. The cells were fixed to the cover slips by two 5-min treatments of cold acetone (−20 C) which was evaporated overnight. The cells were rehydrated in 2 ml of water for 15 min, and the cover slips were removed from the Leighton tubes, rinsed in water, and mounted on a slide in 90% glycerol in 0.01 M phosphate buffer, pH 7.5. Those cover slips showing a group or groups of infected cells when examined with a light microscope were scored as positive, and the $TCID_{50}$ titer was calculated.

Plaque method for the assay of infectivity. Leighton tubes were seeded with 2×10^5 cells/ml and incubated for 24 h. The cells were inoculated and fixed 3 days postinfection (p.i.) when confluency was reached. The cover slips were examined, plaques were counted, and PFU were calculated. The procedure was essentially the same for the 5-day assay except that the initial seeding density was 5×10^4 cells/ml.

Particle-infectious unit ratio. The samples were prepared by mixing titrated nonoccluded virus, polystyrene latex spheres (2.5×10^{11} spheres/ml), uranyl acetate (1% wt/vol), and bovine serum albumin fraction V (1% wt/vol) in a ratio of 2:2:2:1. A single drop was added to a Formvar-coated 200-mesh copper grid, and the excess was drained after 2 min. After a brief rinse in water and drying, the grid was examined in an AEI EM6B electron microscope at an accelerating voltage of 60 kV. Several grids were examined per sample, and photographs were taken of selected fields at a magnification of ×10,000. The polystyrene latex spheres had a mean diameter of 88 ± 0.8 nm (Dow Chemical Co.).

Growth cycle and lateral transmission of infectivity. One 25-cm^2 Falcon flask was seeded for each experiment with 2.5×10^5 cells/ml and incubated for 24 h. The cells were infected at the appropriate MOI with an adsorption period of 1 h. The culture was washed three times with 5 ml of medium and incubated with a final 5 ml. At each interval tested, the medium was removed and pelleted at $1,000 \times g$ for 10 min to bring down any floating cells. The supernatant was stored frozen for titration, and 5 ml of fresh medium was added to the centrifuge tube, mixed, and returned to the culture vessel.

RESULTS

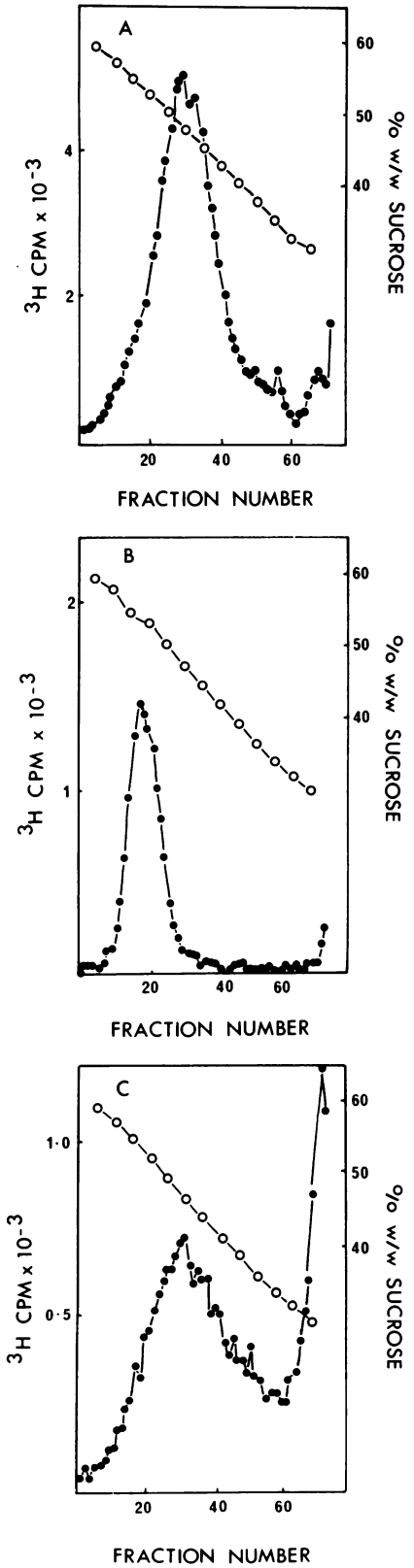
Purification. Consistent profiles were observed when fractions from the purification procedure were analyzed by quasi-equilibrium centrifugation through linear sucrose gradients. A reproducible pattern was seen when the 30,000 *g* pellet of the nonoccluded virus fraction was analyzed (Fig. 1A). The virus banded as a reasonably homogeneous peak at 47 to 49% (wt/wt) sucrose, and there was little radioactive material left at the top of the gradient, suggesting that the purification procedure had adequately separated the virus from cellular debris.

A homogeneous peak was found at a 54 to 55% (wt/wt) sucrose level when the 10,000 *g* pellet of the polyhedra fraction was analyzed. Considerable cellular debris, however, also entered the gradient, and as a result the polyhedra were collected and recentrifuged. The profile of the second cycle of centrifugation clearly indicated that the polyhedra were well separated from the contaminating material that was left at the top of the gradient (Fig. 1B).

If the purified polyhedra were subjected to an alkali dissolution procedure (Harrap and Longworth, in press) and the 30,000 *g* pellet that was obtained was similarly analyzed, a characteristic profile was observed (Fig. 1C). The occluded virus appeared to be quite heterogeneous when compared with the profile of nonoccluded virus (Fig. 1A). Moreover, the occluded virus profile was one of multiple bands over a 35 to 52% (wt/wt) sucrose range, and substantial radioactivity was also detected at the top of the gradient, indicating, perhaps, that degradation of the virus had occurred as a result of the dissolution procedure.

Temperature optimum. Cultures were infected with NPV and incubated at six different temperatures (17, 21, 23, 27, 30, and 37 C). They were scored daily for 6 days for the percentage of infected cells with polyhedra in their nuclei. Since the input virus inoculum was adjusted to yield 20% infection when incubated at 27 C, it was possible to determine the temperature optimum for the replication of the NPV and for the lateral transmission of the infectious material from cell to cell.

The cultures incubated at 27 C had the highest percentage of their cells infected when scored one day p.i., and, therefore, 27 C was the optimal temperature for the replication of NPV (Fig. 2). The 27 C cultures reached a 100% level of infection within 3 days p.i., and, hence, the temperature was also optimal for the lateral transmission of infectivity. The results of the cultures incubated at 30 C resembled those of the 27 C cultures, although the latter were marginally more efficient. The cultures incubated at 17 and 37 C gave similar results, but 37 C was deleterious to the cells, as they rarely remained viable for more than 3 days. Nevertheless, polyhedra were detected in cells incu-



bated at 37 C, suggesting that the virus could replicate at the higher temperature.

Assay of infectivity. The distinct CPE observed in the nuclei of NPV-infected cells was used as the basis for the scoring of the end-point dilution titrations of the virus. Cultures were seeded with a cell density which formed a confluent monolayer of cells on the cover slip after 5 to 7 days of incubation at 27 C, thus facilitating the scoring of infected cultures as several cycles of virus replication had occurred. Leighton tubes were used, instead of the common tube method, because the improved optics

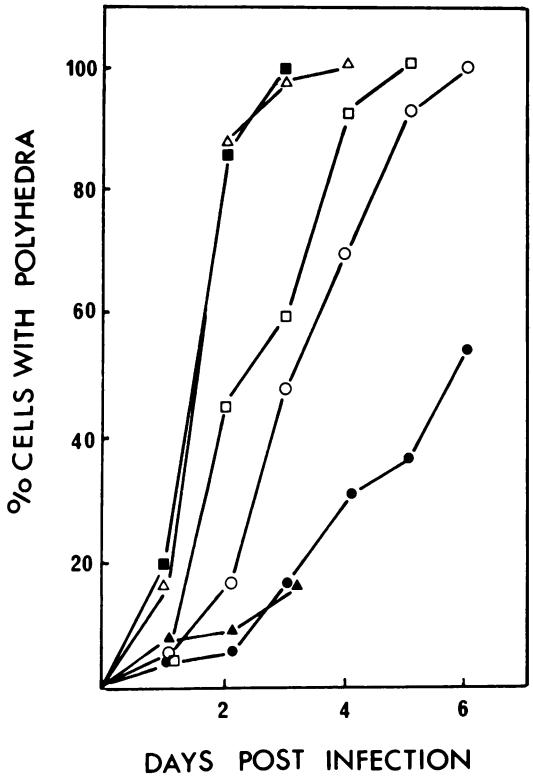


FIG. 2. *Temperature optimum study for the replication and lateral transmission of infectivity of the NPV in its homologous, S. frugiperda continuous cell culture. The cultures were infected with inocula where 20% of the cells exhibited the characteristic CPE when incubated for 24 h at 27 C. The cultures were inoculated and incubated at the indicated temperatures (± 1 C) and scored daily for the percentage of cells exhibiting a CPE. Symbols: ●, 17 C; ○, 21 C; □, 23 C; ■, 27 C; △, 30 C; and ▲, 37 C.*

FIG. 1. *Sucrose equilibrium density gradients representing the characteristic profiles observed when the nonoccluded virus (A), polyhedra (B), and occluded virus (C) fractions were analyzed. Sedimentation is right to left. Symbols: ●, trichloroacetic acid-precipitable ^{3}H counts/min and ○, percent wt/wt sucrose.*

allowed the CPE to be readily distinguished. The cover slips, of course, could be fixed and stored as a permanent record of the virus titration. Cultures that were infected with a high MOI did not become confluent, and the number of cells present at inoculation was unchanged, suggesting that cell division did not occur after infection. Cultures that were infected with a low MOI, however, were confluent with groups or plaques of infected cells randomly distributed in the monolayer. Single cells exhibiting CPE were also found, but plaques were always seen when the entire monolayer was examined. Cultures, therefore, were scored positive when a group of three or more cells with CPE was observed.

The appearance of plaques in cultures infected with a low MOI suggested that the end-point dilution method for the assay of infectivity also presented a possible plaque assay system when the plaques were counted, rather than the culture simply scored as infected or uninfected. Figure 3A represents the dose-response data when the total number of plaques was compared with the virus dilution 5 days p.i. A comparison was made between the observed data and the expected data which was calculated from the known $TCID_{50}$ titer of the inoculum assuming 0.7 $PFU/TCID_{50}$. A comparison of the slopes of the two regression lines revealed that the slope of the observed data was 3.5 times greater than the slope of the expected data.

Two plaque types, however, were distinguishable when the monolayers were reexamined. A small plaque was a group of three or more infected cells with a plaque diameter less than 100 μm (Fig. 4), and those plaques greater than 100 μm were designated as large plaques (Fig. 5). Figures 3B and C, respectively, represent the dose-response data for the small and large plaques compared with the expected data. The slopes of the regression lines through the observed and expected data points revealed that the small plaque data were twice that of the expected slope, whereas the large plaque and

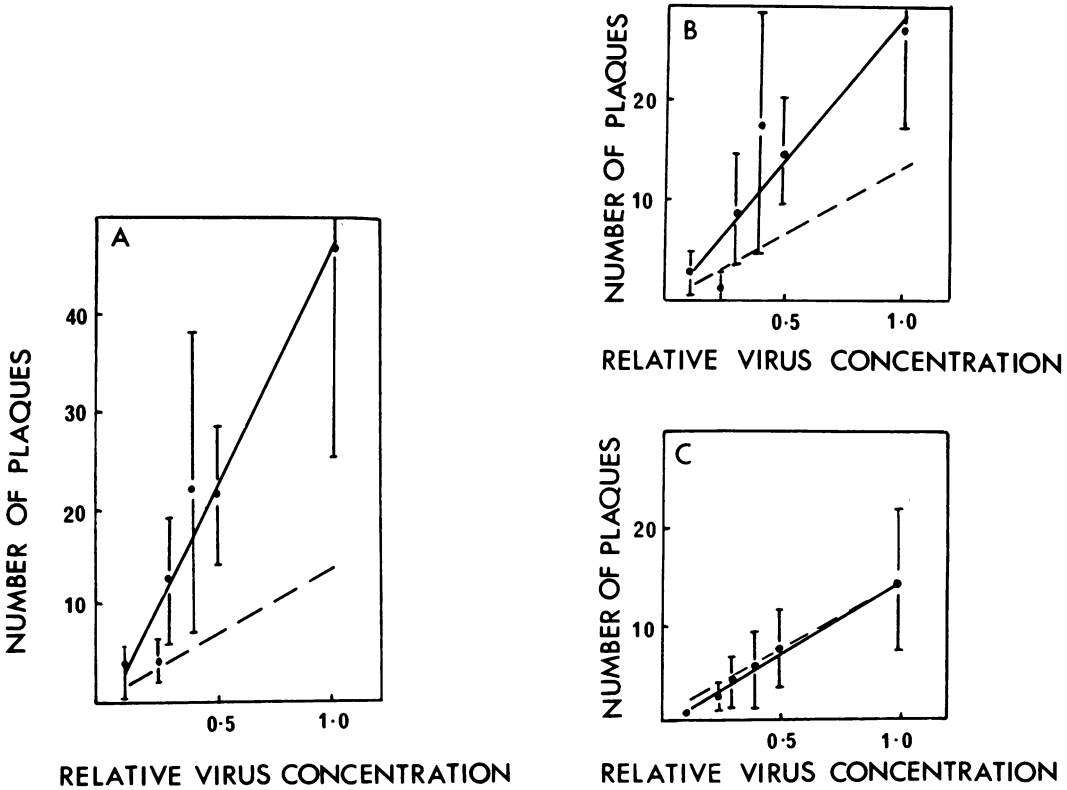


FIG. 3. *Dose-response curve experiments scored 5 days p.i. (A) represents the total plaques seen versus the relative virus dilution. When the cover slips were rescored for small (B) and large plaques (C), only the large plaque data corresponded with the expected result based upon the $TCID_{50}$ titer of the inoculum. Symbols: ●, data points with standard deviations of the five scored cultures; ——, calculated regression line through data; and - - - -, expected regression based upon the $TCID_{50}$ titer, assuming 0.7 $PFU/TCID_{50}$.*

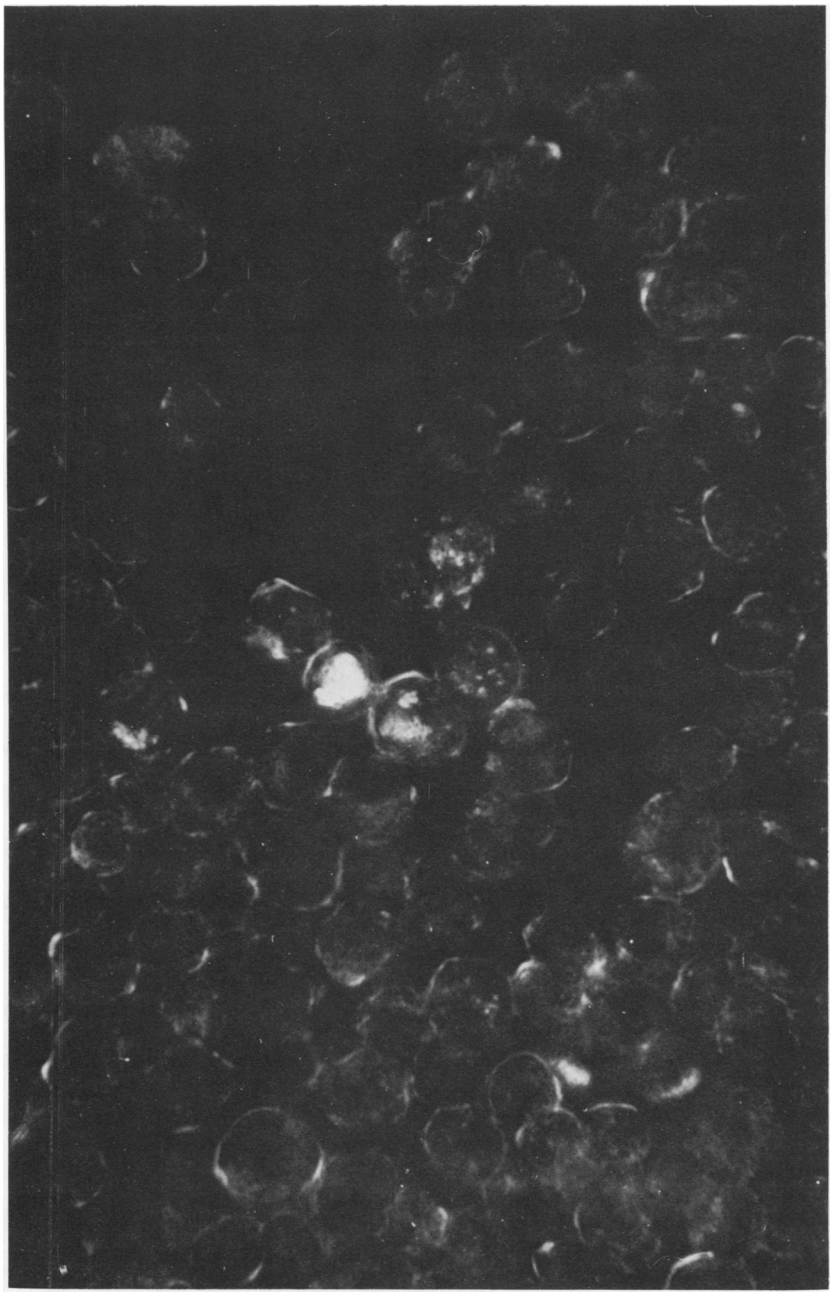


FIG. 4. *Photomicrograph of a characteristic small plaque that is seen when cultures are infected with the NPV at a low MOI and scored 5 days p.i. Polyhedra appear as refractile inclusions in the nucleus of infected cells, resulting in a readily distinguishable CPE. (Magnification* $\times$ *2,350).*

the expected slopes were essentially equivalent. The expression of the two plaque types implied that two phenotypes were present in the virus inoculum. Yet, the dose-response data (Fig. 3A, B, and C) were contrary to this and suggested that the small plaques were derived from the large plaques as a result of a secondary, lateral transmission of infectivity.

No distinction of plaque size was made when the dose-response experiment was repeated, but scored 3 days instead of 5 days p.i., because the plaques were all approximately 100 μm in diameter (Fig. 6).

Particle-infectious unit ratio. Table 1 illus-

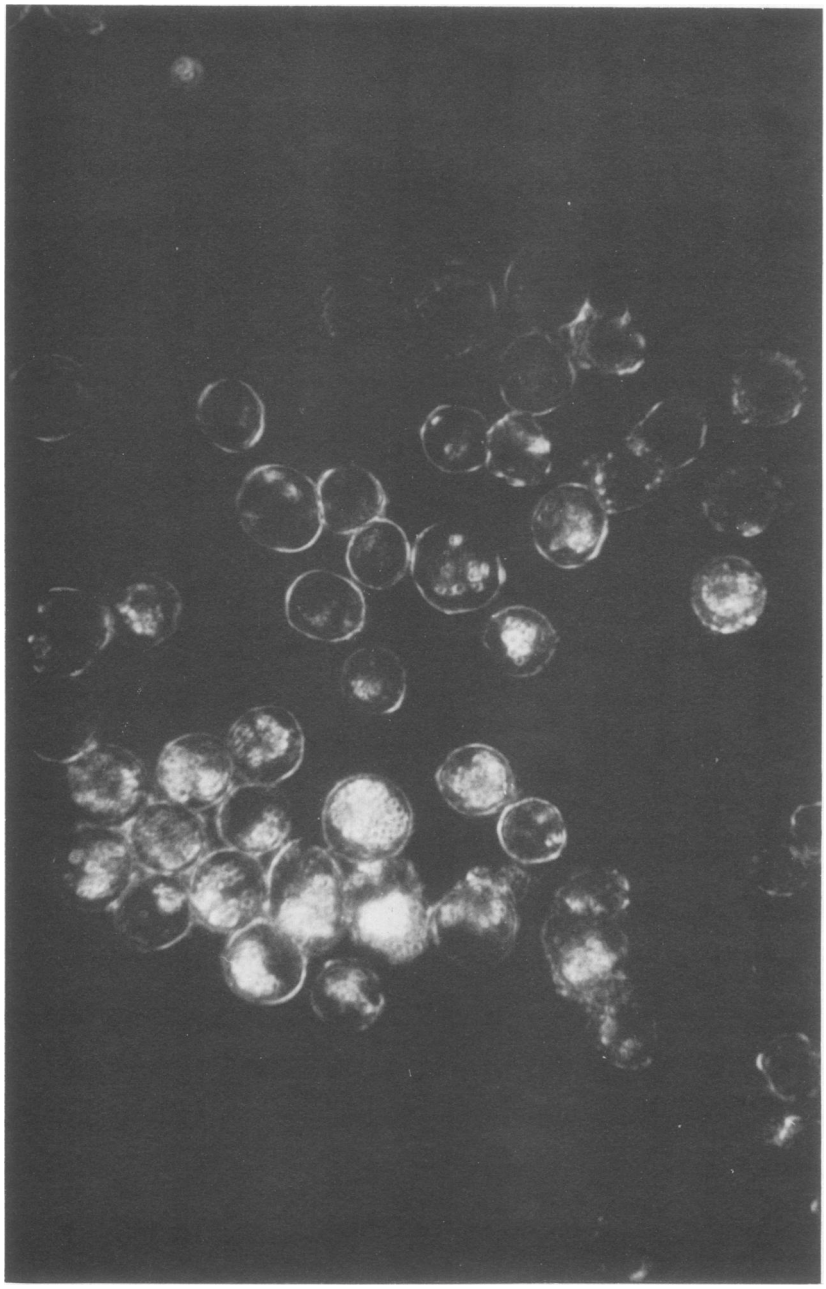


FIG. 5. *Photomicrograph of a characteristic large plaque that is seen when cultures are infected with the NPV at a low MOI and scored 5 days p.i.*

trates the complete particle count data in which three determinations of the ratio were made from three separate preparations of titrated nonoccluded virus that was stored at 4 C. The means and their standard deviations are presented for each of the three samples, and a final calculation from the pooled data gave a mean value of 186 ± 124 particles/$TCID_{50}$ with a standard error of 21%. A corresponding value of 266 ± 177 particles/PFU was calculated using the relationship of 0.7 PFU/$TCID_{50}$.

Growth cycle and lateral transmission of infectivity. Figure 7 represents the growth cycle of extracellular infectivity that was produced when a cell culture was infected with NPV with an MOI of 4.5. The titer of the released material

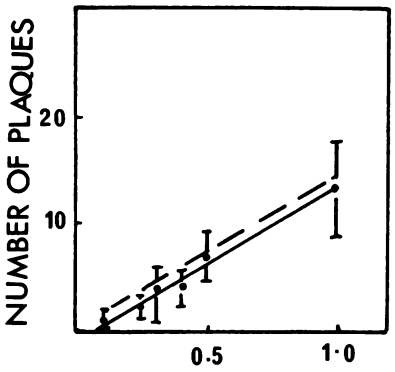


FIG. 6. *Dose-response curve experiment scored 3 days p.i. The plaques that were seen were all approximately the same size, i.e., approximately 100 μm in diameter. Symbols: as described in Fig. 3.*

was determined at intervals and an accumulated titer was calculated. Infectious material was released from the infected cells before 24 h p.i. and it approached a maximal titer 4 days p.i. The growth cycle experiment was repeated over the initial 24-h period to determine whether elution of virus adsorbed to the surface of the cells had occurred and/or to define more precisely when infectious material was released. Figures 8A, B, and C, respectively, represent the early portion of the growth cycle when cultures were infected with MOI's of 0.2, 4.5, and 25 $TCID_{50}$ per cell. Elution of the virus did not appear to significantly contribute to the observed titer. Moreover, infectivity was found extracellularly 12 h p.i. regardless of the MOI.

DISCUSSION

Virus rods or nonoccluded virions are formed in the cell nucleus during the cycle of the NPV replication in the insect. As the infection proceeds, an outer envelope surrounds the virus or groups of virus which then become incorporated into a matrix of the polyhedral protein (5–7). A similar pattern of viral morphogenesis has been observed in NPV-infected cell cultures (unpublished data). Polyhedra and nonoccluded virus can be found intra- and extracellularly as the replicative cycle progresses. In the results reported here purification of these two viral entities has been effected, and there are several observations which substantiate this claim. For example, the homogeneity of the profile of nonoccluded virus (Fig. 1A) suggested that a population of unbundled virions was isolated. The binding density of the nonoccluded virus (Fig. 1A) suggests that nucleocapsids, in fact, were isolated, but the data is inconclusive and there may be some envelope material associated with the nonoccluded virus. This material, however, was used for the calculation of the particle-infectious unit ratio, and the preparations consistently revealed well-dispersed unenveloped nucleocapsids. The profile (Fig. 1C) that was seen when purified polyhedra were disrupted with alkali releasing occluded virus (both enveloped and unenveloped nucleocapsids) is similar to other multiply embedded NPV of this type (4, 6; Harrap and Longworth,

TABLE 1. *Particle to infectious unit ratio*

Sample	Spheres / Particles	Particles / ml × 10^8	Particles / $TCID_{50}$
1	6.4	392.6	278.4
	3.1	813.2	576.7
	7.7	328.0	232.6
	10.3	246.8	175.0
	6.5	386.5	274.1
	6.7	376.1	266.7
	9.1	276.7	196.2
	6.3	403.3	286.0
	6.0	421.7	299.1
	4.8	520.5	369.2
			295 ± 113
2	26.5	95.5	124.6
	11.0	230.0	300.3
	40.0	63.3	82.6
	12.3	206.5	269.6
	22.0	115.0	150.1
	9.3	271.1	353.9
	16.6	152.4	199.0
	27.0	93.7	122.3
	15.3	165.0	215.4
	7.0	361.4	471.8
	37.3	67.9	88.7
			216 ± 123
3	9.8	257.3	134.0
	15.3	165.9	86.4
	9.8	258.2	134.5
	21.7	116.8	60.8
	16.3	154.9	80.7
	9.2	276.0	143.8
	11.7	216.9	113.0
	21.3	118.6	61.8
	10.5	241.0	125.5
	15.6	162.2	84.5
	28.0	90.4	47.1
	16.5	153.3	79.9
	20.3	124.4	64.8
	15.0	168.7	87.9
	19.2	132.0	68.8
			92 ± 31
Total	186 ± 124 Particles/$TCID_{50}$		

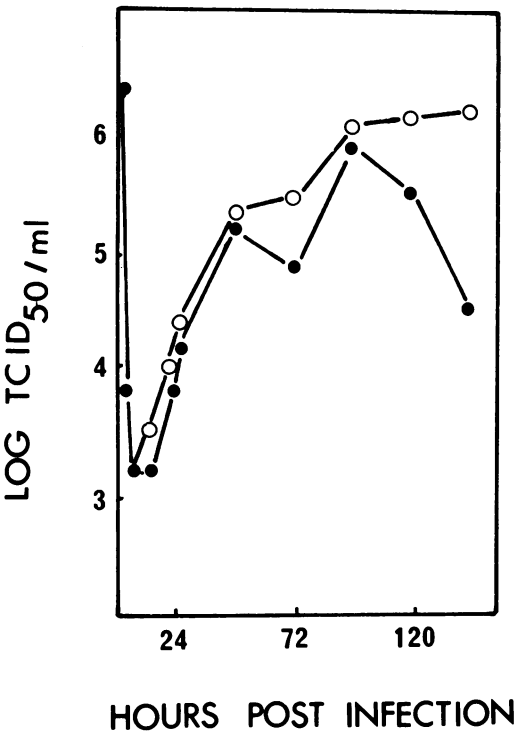


FIG. 7. *Growth curve of the nuclear polyhedrosis virus in cell culture. A culture was infected at an MOI of 4.5 $TCID_{50}$/cell, and the extracellular infectivity was monitored at the indicated intervals. Symbols: ●, log $TCID_{50}$/ml measured at each interval and ○, accumulated log $TCID_{50}$/ml calculated for each interval.*

in press). The alkali treatment must have disrupted or removed the envelopes from some of the occluded virus, since a correlation of peak fractions was observed between nonoccluded virus and occluded virus. Moreover, radioactive material was found at the top of the gradient, indicating that the procedure had affected the integrity of the enveloped nucleocapsids.

The nonoccluded virus was used as the source of inoculum for several reasons. For example, the alkali dissolution procedure was eliminated as a purification step and the yield of nonoccluded virus approached ten times that of occluded virus. There is also evidence suggesting that the dissolution procedure reduces the infectivity of the virus (unpublished data).

The end-point dilution and plaque methods for the assay of infectivity offer distinct advantages over preexisting methods. The end-point dilution method using primary cultures as the assay system (17) has the obvious drawback of the difficulty inherent in the establishment of such cultures. The quantal response method, in which the percentage of uninfected cells were scored 24 h p.i. (1), gave conservative estimates of the virus titer since the population doubling time of the cells used was 16 h (9). The percentage of uninfected cells, therefore, was increasing. The plaque assay method, employing a 0.6% methylcellulose overlay (10), raises several questions when compared with the data presented in this paper. For example, the authors reported (10) that two plaque types, the "many polyhedra" plaque (MP) and the "few polyhedra" plaque (FP), were found. The MP plaques were larger when compared with the FP plaques, and there were 4 to 12 times more FP than MP plaques when the input inoculum gave 40 to 60 total plaques per assay. When the FP and MP plaques were cloned and grown in the insect, purified, and reinoculated for plaque assay, both FP and MP were recovered from each clone. Moreover, a dose-response experiment is presented where only two points with a 10-fold difference were used to indicate that the response was linear. The reported cloning data implied that two plaque types were not isolated. Either the passage through the insect had some unexplained effect, the clones were cross-contaminated, or perhaps the FP and MP plaque types were not true phenotypes of the possible heterogeneity of the virus inoculum. Perhaps the two plaque types exist, but their plaque assay method does not prevent secondary, lateral transmission of infectivity from the MP plaque, and difficulty, therefore, would be encountered when attempting to isolate or pick a "true" FP plaque from the "false" FP plaques that may also be present. The analogous situation of two plaque types existed in the *S. frugiperda* plaque assay system when assays were scored 5 days p.i. However, evidence has been presented which demonstrates that the slopes of the plaque and expected data are equivalent when the assay is scored 3 days p.i., suggesting that a secondary, lateral transmission of infectivity had occurred in the 5-day assay. The small plaques, therefore, were derived from the large plaques. The 3-day assay suggests that a single virus particle can initiate the infection of the cell. It also confirms the relationship of 0.7 PFU/$TCID_{50}$ and further substantiates the accuracy of these infectivity measurements.

The growth cycle data demonstrates that infectious material was released from infected cells 12 h p.i., reaching a maximal titer 4 days p.i. Cytolysis did occur to a limited extent 2 to 3 days p.i., and perhaps the adsorption of virus to newly exposed receptor sites accounts for the

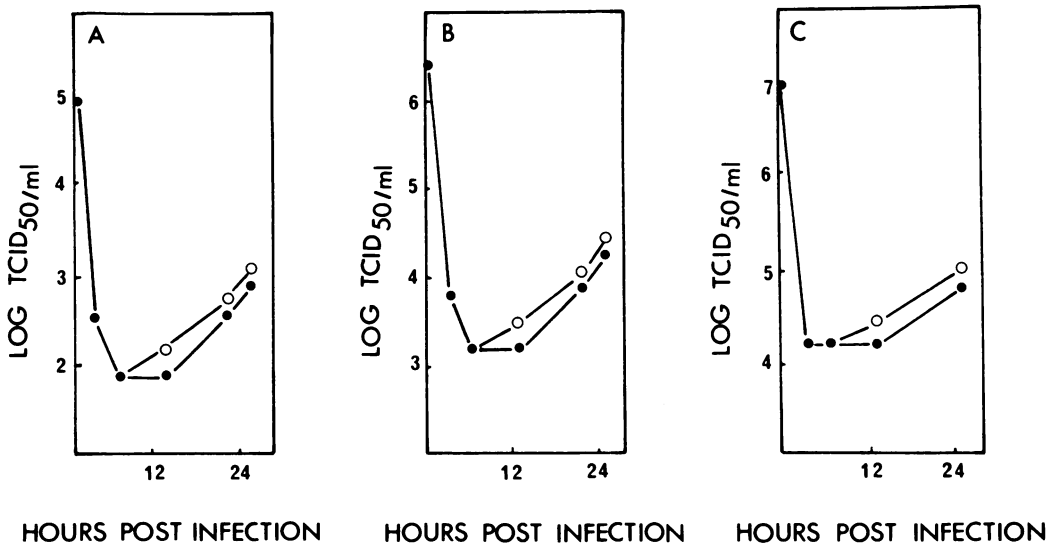


FIG. 8. *Growth curves of the nuclear polyhedrosis virus in cell culture over a 24-h interval. Cultures were infected at an MOI of 0.2 (A), 4.5 (B), and 25 (C) $TCID_{50}$/ml, and the extracellular infectivity was monitored at the indicated intervals. Symbols: as described for Fig. 7.*

drop in the interval titer. It is interesting to note that lateral transmission of infectivity occurred 1 to 2 days before any lysis was observed. Virus, however, has been detected in the nuclei of infected cells 12 h p.i. and has been observed in the process of budding from the cell surfaces (unpublished data).

Several conditions were established for the optimal replication of NPV in a continuous cell culture of *S. frugiperda*. The temperature optimum for the growth of the cells of 27 C was also the optimal temperature for the replication of the virus. In preliminary experiments where cultures were maintained at confluency on medium containing 2% fetal bovine serum instead of 10% serum and infected, no CPE was observed (unpublished data). The virus may replicate in these cultures without producing a CPE, due to the altered conditions of the medium. Yet, the observation that once the cell is infected, cell division is halted may suggest that the cells must be actively dividing or in their logarithmic growth phase for infection to proceed. Moreover, this phenomenon may explain why plaques (Fig. 4 and 5) are found without the use of an overlay that would normally impede the lateral transmission of infectivity. The seeding density of the cells, therefore, is important in this plaque assay method. The cultures must be seeded to allow the development of a plaque, and, at the same time, the uninfected cells must grow and approach confluency which then, apparently, renders them incapable of producing a CPE.

The number of $TCID_{50}$ produced per cell can be calculated from the growth cycle experiment. It must be noted, however, that the titers of virus stored at 4 C as compared with those stored at −20 C may differ by half a logarithm (unpublished data), and the total $TCID_{50}$ produced, therefore, was 1.6 to 5 × 10^6 $TCID_{50}$/ml. Since 5 × 10^5 cells/ml were used, a value of 3 to 10 $TCID_{50}$/cell can be calculated. Table 2 represents the typical yields of virus that were produced under the described conditions. The calculations are approximations, but they probably represent a reasonable estimate of the yields of NPV that can be expected from infected cell cultures. Nevertheless, it becomes readily apparent that insect cell systems will

TABLE 2. *Virus production*

Values	Yield		
	Polyhedra	Nonoccluded virus	$TCID_{50}$
Per liter flask[a]	7 × 10^{8b}	9 × 10^{9c}	10^8–5 × 10^8
Per cell[d]	12–40	150–500	3–10

[a] Conditions: seed, 5 × 10^6 cells; MOI, 0.01 (5 × 10^4 $TCID_{50}$); harvest, 7 to 10 days.

[b] Polyhedral protein: 0.7 mg.

[c] Nonoccluded virus protein: 0.3 mg.

[d] Calculation based on the observations that the liter flask was confluent (5 × 10^7 cells), a ratio of 4 polyhedra:50 virions: $TCID_{50}$ was produced, and 3 to 10 $TCID_{50}$ were produced per cell (growth cycle data).

provide a feasible means by which the replication of these viruses can be investigated.

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