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Comparison of Biophysical and Morphological Properties of Occluded and Extracellular Nonoccluded Baculovirus from In Vivo and In Vitro Host Systems

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Electron microscopic examination and buoyant density profiles of nonoccluded *Rachiplusia ou* and *Autographa californica* nuclear polyhedrosis viruses purified from both infectious insect hemolymph and cell culture medium revealed that the viruses are enveloped, single nucleocapsids. The envelopes exhibited variation in the amount and the degree of fit with regard to the nucleocapsids. This was determined by: (i) electron microscopic observations of virus budding from the surface of infected cells; (ii) electron microscopic observations of negatively stained preparations of pelleted, highly purified, nonoccluded enveloped particles; and (iii) the resolution and density distributions of nonoccluded virus in sucrose gradients after centrifugation to equilibrium; all were compared with virus extracted from polyhedra. Peplomers, observed on the surface of enveloped nucleocapsids of nonoccluded virus, are not associated with polyhedra-derived virus. Density gradient analysis indicated that virus from insect hemolymph and culture medium exhibited similar densities of approximately 1.17 to 1.18 g/ml. This is significantly different from the buoyant density of an alkali-liberated, enveloped single nucleocapsid (1.20 g/ml). Results of this study show that the nonoccluded forms of two nuclear polyhedrosis viruses from two different sources, hemolymph and cell culture, are similar with regard to several morphological and biophysical characteristics but are quite different from the alkali-liberated, polyhedra-derived form of the virus.

The use of lepidopterous cell lines for the study of the kinetics and biochemistry of baculovirus replication is a comparatively recent development in insect virology. One of the significant observations made in early studies is that virus, as isolated from purified polyhedra, is relatively noninfectious in cell culture, whereas extracellular, nonoccluded virus recovered from the hemolymph of infected insects or medium from infected cell cultures is highly infectious. There are preliminary reports on the characterization of the nonoccluded infectious virus, but some are contradictory. Thus the infectious entity has been described as being DNase sensitive (6), a nucleocapsid (7, 8, 10), or possibly an enveloped nucleocapsid (3, 8, 9). Further, Zherebtsova et al. (15) detected subviral infectivity in virus preparations from *Galleria mellonella*.

In this study, we have attempted to clarify the nature of the nonoccluded infectious virus by making biophysical and morphological comparisons of the nonoccluded infectious material from insect hemolymph and cell culture medium (CCM) with virus derived from inclusion bodies. The viruses used were the *M*NPVs (*M* is used to designate many nucleocapsids common to a single envelope as compared to enveloped single nucleocapsids, *S*NPV) of *Rachiplusia ou* (Ro*M*NPV) and *Autographa californica* (Ac*M*NPV). The information obtained indicates that both the Ro*M*NPV and Ac*M*NPV viruses from these two sources are similar in that they are predominantly loosely enveloped single nucleocapsids. These extracellular forms of the virus are different from the enveloped nucleocapsids purified from occluded virus with regard to buoyant density and degree of envelope fit, surface structure, and average number of nucleocapsids per envelope.

MATERIALS AND METHODS

Cell culture. The continuous cell line of *Trichoplusia ni* TN-368 was obtained in passage 57 from W. F. Hink, Ohio State University, in June 1974. Since then the cells have been passed routinely three times per week in disposable plastic tissue culture flasks, with the initial concentration being 10^5 to 2×10^5 cells/ml. Daughter cell lines were developed from

isolated single cells of TN-368 (L. E. Volkman and M. D. Summers, *In Proceedings of the IVth International Conference on Invertebrate Tissue Cultures*, in press), and these cloned lines were handled in the same manner as TN-368. The cultures were grown at 28 C and the medium used was TNM-FH (4). Antibiotics were not used for routine maintenance of the cells. The population doubling time for all the lines was 15 to 17 h.

Virus. Initially Ac*M*NPV was supplied by P. V. Vail and W. F. Hink in the form of infectious tissue culture medium. TN-368 cells infected with this medium yielded extracellular virus as well as polyhedra, which were in turn used to infect *T. ni* larvae for the production of infectious hemolymph. Ro*M*NPV was obtained in the occluded form from C. Y. Kawanishi. *T. ni* larvae infected with the polyhedra yielded the infectious hemolymph, which was used subsequently to infect TN-368 cells for the production of nonoccluded virus.

Plaque assay of infectious virus. For the plaque assay (5, 14), log-phase TN-368 or TN-368 clone 10 cells (TN-368-10) were seeded into 30-mm Corning plates at 3.5×10^6 cells/plate in 2 ml of medium. Cells were allowed to attach for 1 to 2 h. The medium was carefully removed, and the cells were inoculated with a 0.1-ml sample/plate containing between 50 and 250 PFU. Plates were rocked every 10 min for 1 h and then overlaid with 0.9% methylcellulose in TNM-FH containing 200 μg each of penicillin and streptomycin per ml and 5 μg of Fungizone per ml.

Density gradient analysis. Sucrose gradients for the assessment of the distribution of viral biological activity were constituted in TNM-FH and were prepared with a density range of 1.1 to 1.3 g/ml over a 0.5-ml cushion of saturated sucrose. Centrifugation was conducted at 60,000 rpm (SW65 rotor) and 10 C for 2 or 14 h, with little differences in the infectivity distributions observed relative to the period of centrifugation.

Sucrose gradients for visual comparison of the distribution of culture-derived extracellular virus and alkaline-liberated virus were constituted in 0.01 M Tris, pH 7.8, 0.01 M EDTA, with a density range of 1.1 to 1.3 g/ml for the former and 1.17 to 1.3 g/ml for the latter. The gradients were centrifuged for 4 h at 24,000 rpm (SW27 rotor) at 4 C.

Sample preparation for gradient analysis. Enveloped nucleocapsids present in CCM 24 or 72 h postinfection were prepared for characterization by several methods. (i) Infectious CCM was given two cycles of differential centrifugation at 225 × *g* (clinical centrifuge) to remove cells with a minimum of disruption. The 225 × *g* supernatant fluid was immediately layered on sucrose gradients for centrifugation. (ii) For additional clarification the 225 × *g* supernatant fluid was centrifuged at 4000 × *g* (clinical centrifuge), and the supernatant was recovered and immediately centrifuged at 12,000 × *g* (12G) for 45 min. The CCM-12G pellet was allowed to resuspend in 0.5 ml of TNM-FH overnight and then was layered onto sucrose gradients for centrifugation. (iii) The CCM-12G supernatant was immediately centrifuged at 100,000 × *g* for 45 min, and the pellet (CCM-100G) was allowed to resuspend overnight in 0.5 ml of TNM-FH. Both the 100G pellet and 100G supernatant were layered onto sucrose gradients for centrifugation. All virus fractions were centrifuged to equilibrium as described above. Hemolymph was obtained from infected *T. ni* larvae by removing one or two prolegs and catching the resultant drops of infectious fluid in a test tube containing TNM-FH with antibiotics. The dilution factor was approximately 2 drops/ml of medium. The diluted hemolymph was analyzed directly by density gradient centrifugation without further manipulation.

The gradient centrifugation of 0.5-ml samples of infectious CCM or hemolymph containing about 10^6 PFU did not result in the appearance of any bands of UV-absorbing material. To assay for virus distribution after centrifugation, gradients were fractionated into 0.25-ml fractions with an ISCO density gradient fractionator. Densities of the samples were determined with an Abbe refractometer. The quantitation of PFU per fraction was done by the plaque assay method described earlier.

Enveloped nucleocapsids were liberated from the proteinic crystals by a modification of the usual dilute alkaline saline solubilization procedure (13). After a brief period of solubilization in 0.1 M Na_2CO_3 + 0.05 M NaCl, pH 10.9, the preparation was centrifuged at 2,000 × *g* for 5 min to remove the debris. The supernatant was layered onto sucrose gradients and centrifuged as described above

Electron microscopy. TN-368 cells were prepared for electron microscopy using a modification of a procedure devised by Velma C. Chambers (1). The cells were grown in 30-mm Corning tissue culture dishes. At 36 h after infection, the cell culture medium was carefully removed from the cells and replaced by a 3% glutaraldehyde-acrolein mixture in sodium cacodylate buffer and then were dehydrated and embedded as described by Summers and Arnott (12). Sections were stained with uranyl acetate and Reynolds lead citrate and examined on a Siemens I electron microscope. Virus solutions were prepared for negative staining using a 2% ammonium molybdate solution buffered to neutrality.

RESULTS

Equilibrium banding by centrifugation in sucrose gradients of extracellular infectious virus recovered directly from CCM 24 h after the infection of TN-368, as well as of TN-368-10 cells, reveals that approximately 95% of the infectivity is somewhat homogeneously distributed in a major band, with a density of approximately 1.17 to 1.18 g/ml (Fig. 1). A minor band of infectivity resulting from nucleocapsids is apparent (5%) as recovered from the cushion at the bottom of the gradient. If the TN-368-derived virus is pelleted by centrifugation at 12,000 × *g* (12G) for 45 min and then analyzed by gradient centrifugation (Fig. 2), it retains its relatively homogeneous nature and is somewhat

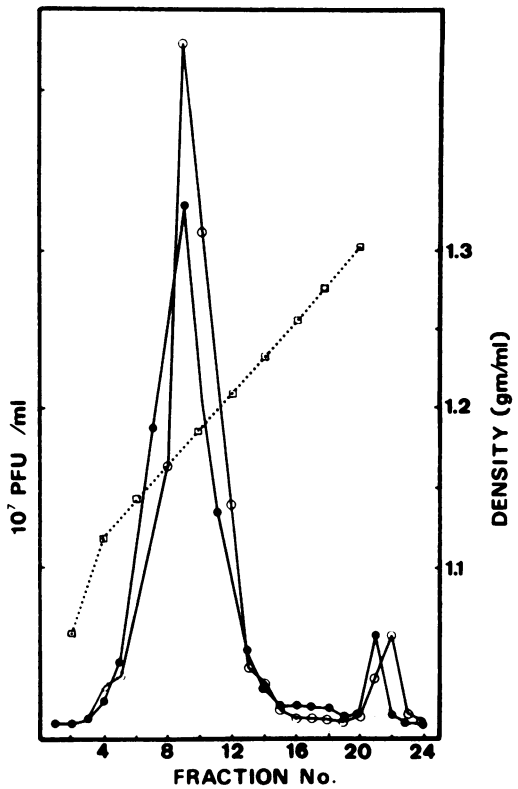


FIG. 1. *Infectivity distribution after centrifugation to equilibrium of CCM from AcMNPV-infected TN-368* (○) *or TN-368-10* (●) *cells. One-half-milliliter samples of CCM recovered from cells 24 h postinfection were centrifuged two times at 225 × g to remove cells and cellular debris. Samples were layered directly onto sucrose in TNM-FH gradients. The gradients ranged in density from 1.1 to 1.28 g/ml and had a 0.5-ml cushion of saturated sucrose. Centrifugation was at 60,000 rpm (SW65 rotor) and 10 C for 2 h. Gradients were fractionated into 0.25-ml samples, and infectivity distribution was determined by plaque assay (5).*

similar to that observed in Fig. 1. If, however, it is pelleted by centrifugation at 100,000 × *g* (100G) for 45 min and analyzed by gradient centrifugation, considerable heterogeneity results, as evaluated by the infectivity distribution (Fig. 2). A greater proportion (approximately 10 to 13%) of the infectivity is in the form of unenveloped nucleocapsid and is found on the cushion. The major component bands at approximately 1.17 to 1.18 g/ml as shown previously; however, there appears to be more infectivity distributed between the major component and the bottom of the gradient. In addition, there is significant infectivity at the top of the gradient in the density region of about 1.1 to 1.13 g/ml, suggesting the presence of subviral components or nonspecific association of virus with membrane material.

A comparison of the infectivity distribution from the 100G pellet of virus grown in TN-368 cells to that of the more gently treated preparations (Fig. 1) suggests that the centrifugation procedure is an undesirable one, giving rise to disruption of the virus and/or aggregation of viral components. In contrast, a comparison of the results of a similar experiment using virus grown in TN-368-10 cells shows (Fig. 3) that there is comparatively little difference between the 12G pellet and the 100G pellet by gradient centrifugation analysis. The reason for the added stability of this viral preparation is not apparent to us at this time.

Figure 4 shows the comparative infectivity distribution from hemolymph of infected *T. ni* larvae and from TN-368 culture medium 72 h after infection. Samples from both were layered directly onto gradients without prior centrifugation. For both sources of infectious material the major component exhibiting infectivity has a

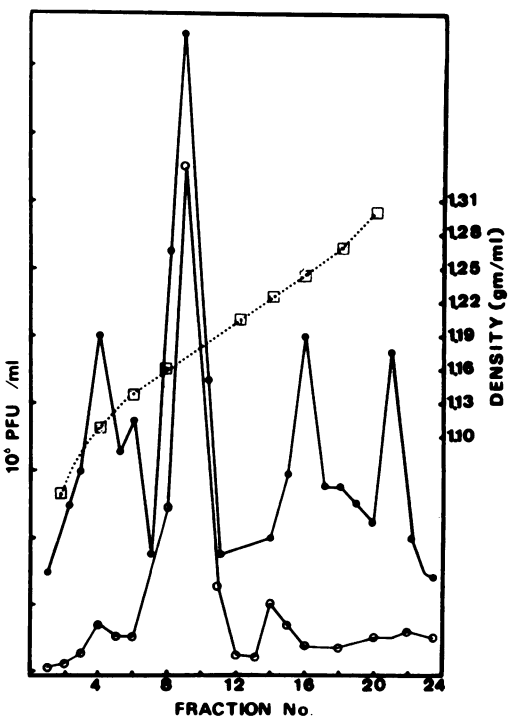


FIG. 2. *Comparative infectivity distribution on sucrose gradients of the 12G* (○) *and 100G* (●) *pellets of extracellular virus (24 h) from AcMNPV-infected TN-368 cells (see text). Centrifugation conditions and method of assay were as previously described (see Fig. 1).*

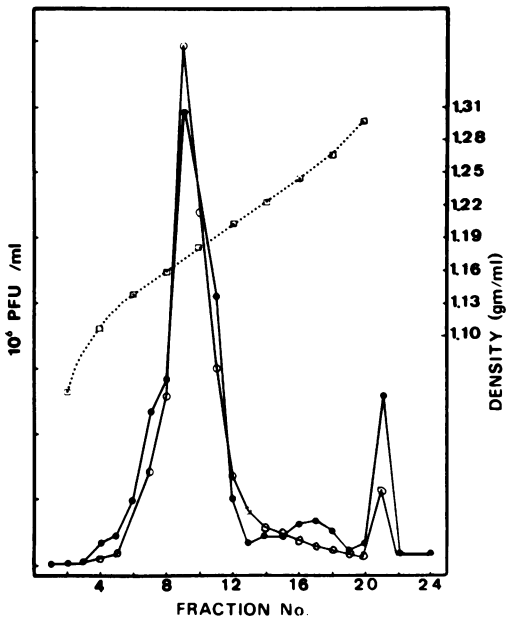


FIG. 3. *Comparative infectivity distribution on sucrose gradients of the 12G (○) and 100G (●) pellets of extracellular virus (24 h) from AcMNPV-infected TN-368-10 cells (see text). Centrifugation conditions and method of assay were as previously described (see Fig. 1).*

mean density of about 1.17 to 1.18 g/ml, with the extremes in those peaks ranging from 1.14 to 1.20 g/ml. A small percentage of the total infectivity from the CCM was observed in higher density regions of the gradient at about 1.25 g/ml. This infectivity in the higher density range from the CCM sample of the 72-h-old infected TN-368 cell culture may represent virus that has partially lost its envelope and/or bundles of nucleocapsids common to a single envelope, which may have been released into the medium by cell lysis. (Cell lysis has been noted in 72-h-old infected cultures.) Although the 72-h infectious CCM banding pattern is similar to the infectious hemolymph pattern only in the density position of its major component, the banding profiles of the infectious material from insect blood (Fig. 4) and of the 24-h-old CCM (Fig. 1) are really quite similar. A comparison of Ro*M*NPV infectivity from CCM and from hemolymph (data not shown) was not significantly different from that of the Ac*M*NPV profiles. A peak of infectivity associated with infectious hemolymph was occasionally observed at the bottom of the gradient (nucleocapsid), suggesting that nonenveloped particles were present in these preparations as well. However, the infectious hemolymph had been stored for some time and had been recovered from insects in an advanced state of infection (4 to 5 days postinfection but prior to death).

A comparative study of the density distribution of alkali-liberated virus and virus derived from CCM indicated that virus from these two sources were significantly different. Alkali-liberated virus exhibited the typical multiplicity of bands observed for *M*NPV viruses (2). The density range for alkali-liberated virus, which exists in units of one nucleocapsid to many nucleocapsids per envelope, was from 1.20 to 1.21 to 1.25 g/ml, as analyzed on both sucrose and CsCl gradients (not shown). A direct comparison of the distribution of infectivity from hemolymph and tissue culture medium containing Ro*M*NPV and alkali-liberated Ro*M*NPV on identical sucrose gradients (Fig. 5) revealed that the major infectious component from the hemolymph and from CCM was of a density significantly less than that of enveloped single nucleocapsids purified from occluded virus. A visual comparison of the sucrose gradient distribution of alkali-liberated Ac*M*NPV with CCM-derived Ac*M*NPV dramatically illustrated and reconfirmed the infectivity data (Fig. 6A and B), i.e., that the infectious entities were indeed physically different.

Since virus recovered from infected culture cells exhibited a heterogeneous distribution compared to the extremely sharp banding patterns characteristic of the alkali-liberated virus, an attempt was made to visualize by electron microscopy whether or not the nature and amount of viral envelope to the nucleocapsid might account for this difference. Cultured cells embedded and fixed at 36 and 68 h showed the presence of budding virus from the plasma membrane (Fig. 7). The association of viral envelope with nucleocapsid during the process of exocytosis was observed to be loose and/or variable in nature. The loosely adhering envelope about the nucleocapsid was seen as well in negatively stained preparations of 12G infectious CCM pellets. The virus shown in Fig. 8A and B is characteristic of a major portion of the particles viewed. It is interesting that virus from cell culture showed some evidence, although not distinct, of having surface projections (viral peplomers; Fig. 8A, B, and C), which is not characteristic of alkali-purified virus (Fig. 10A and B). Figure 9 shows a negatively stained virion from the 12G pellet of hemolymph of Ac*M*NPV-infected larvae. Although disrupted, the particle retained portions of its envelope, which clearly possess surface projections. It should be noted that a large number of enveloped nucleocapsids in the negatively stained

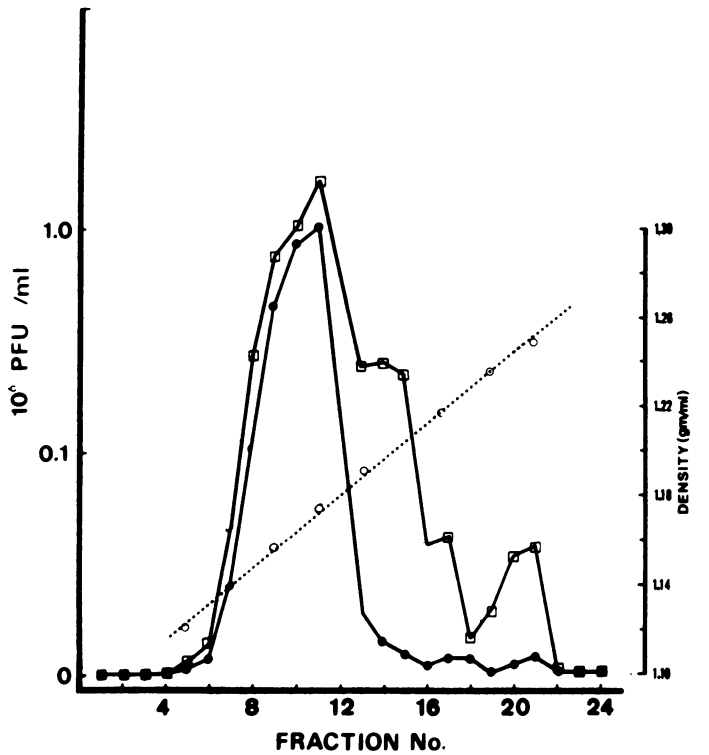


FIG. 4. *Comparative infectivity distribution on sucrose gradients of AcMNPV from hemolymph (●) and from the CCM of TN-368 cells 72 h postinfection (□). For conditions of sample preparation and centrifugation see the text.*

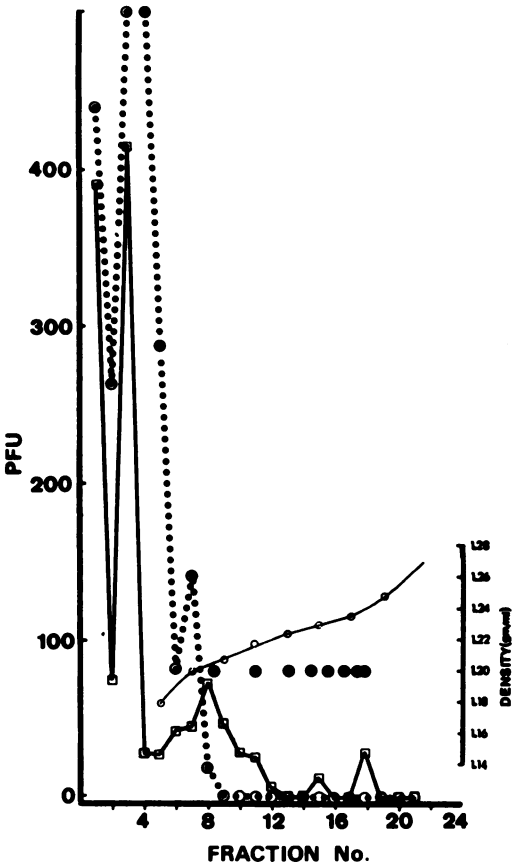


FIG. 5. *Comparative distribution on sucrose density gradients of alkali-liberated RoMNPV from polyhedra (●); (each dot of increasing density = one additional nucleocapsid per envelope); RoMNPV from the CCM of infected TN-368 cells (□); and RoMNPV from the hemolymph of infected T. ni larvae (····). Density range of the gradients is 1.14 to 1.28 g/ml, differing from Fig. 1 through 4. For conditions of sample preparation and centrifugation, see the text.*

preparations showed disruption of the viral envelope unless great care was taken in handling and preparing the grids.

DISCUSSION

The results of this study show that extracellular nonoccluded virus from CCM and from insect hemolymph are similar with regard to buoyant density and surface structure and are very likely different physical entities from the virus obtained by alkali extraction from polyhedra. In these studies the major form of the virus responsible for infection appears to be an enveloped single nucleocapsid. Electron microscopic observations of nucleocapsids budding from the surface of infected virus reveal that the envelopes of culture-derived extracellular virus are very loosely associated with the nucleocapsids. We found these envelopes to be very fragile, as accessed by centrifugation techniques. Henderson et al. (3), in characterizing *T. ni* MNPV, suggested that the envelopes of these particles were fragile as well, because great difficulty was encountered in observing completely enveloped virus particles in negatively stained electron microscopic preparations. These fragile, loose-fitting envelopes may well be responsible for the relatively broad distribution in sucrose gradients of the infectious particles.

Since hemolymph-derived virus had a buoyant density and a density gradient distribution pattern similar to the culture-derived extracellular virus, and since negatively stained preparations revealed partially enveloped particles (indicating the envelopes are fragile) with surface projections, it is not unreasonable to speculate that the hemolymph-derived virus is also loosely enveloped. Indeed, Summers (11) has shown that granulosis virus, which buds from midgut cells into the hemolymph of infected *T. ni* larvae, is loosely enveloped.

High-speed centrifugal pelleting of nonoccluded virus can introduce physical heterogeneity into virus preparations, as shown in this report; the preparation can exhibit infectious forms of a greater density than the major component at 1.17 g/ml. Heterogeneity is also seen in nonoccluded, unpelleted virus from 72-h-old infectious CCM, indicating that there may be some natural disruption of viral envelope with increased time in culture at 28 C. This increased physical degeneration of virus with time at 28 C can be correlated with a decrease in biological activity reported elsewhere (14). Alternatively, the heterogeneity may be due, in part at least, to bundles of nucleocapsids that could have been released into the medium by cell lysis.

Henderson et al. (3) demonstrated the buoyant density of the enveloped particle of an NPV of *T. ni* in cesium chloride to be 1.26 g/ml, with the range of density for infectious material being 1.26 to 1.35 g/ml. This density range is much broader than that achieved in our studies. They also reported that they had done electron microscopic studies in which they observed the presence of only partly, never completely, enveloped nucleocapsids. Similar observations were made in our study if preparations were not carefully handled or if acidic negative stains were used.

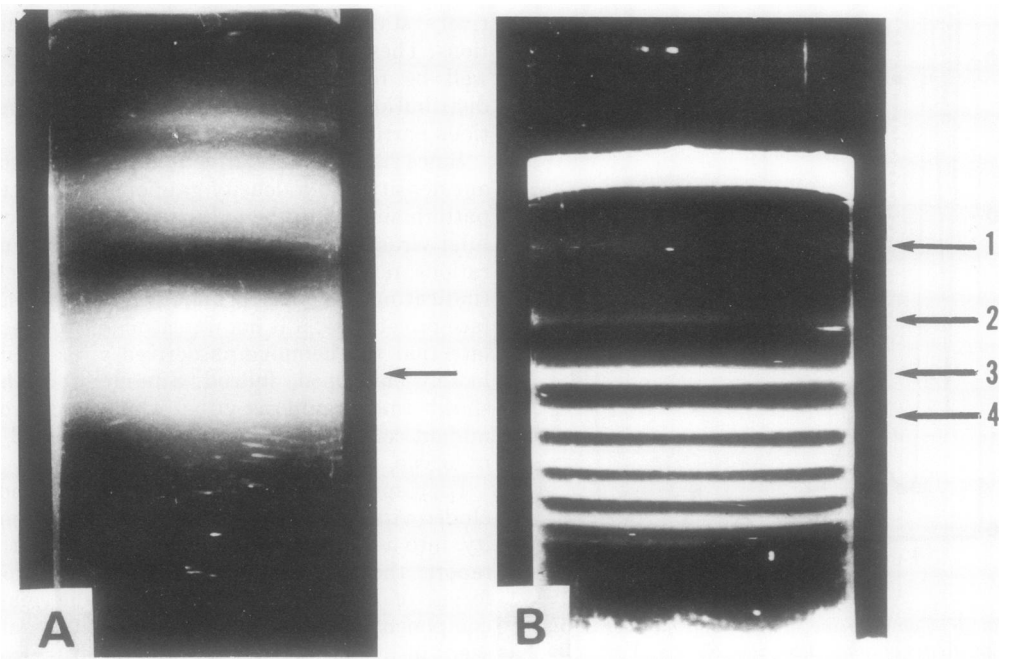
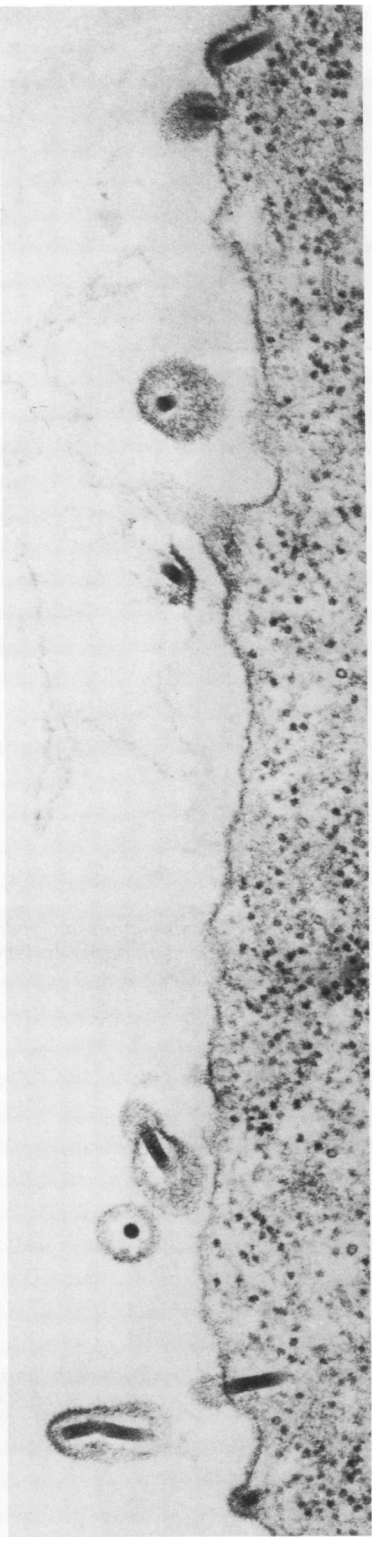


FIG. 6. *Comparison of CCM virus and alkali-liberated virus distribution in sucrose gradients. (A) CCM virus from AcMNPV-infected TN-368-13 cells (24 h) was concentrated by centrifugation at 12,000 × g for 45 min. The pellet was resuspended in 0.01 M Tris, pH 7.8, 0.01 M EDTA and was layered onto a sucrose gradient in that buffer with a density range of 1.1 to 1.3 g/ml and centrifuged for 4 h at 100,000 × g. The virus band of enveloped nucleocapsid (confirmed by electron microscopy) exhibits a density of 1.17 to 1.18 g/ml (indicated by arrow). (B) Alkali-liberated AcMNPV was layered onto a sucrose gradient with a density range of 1.17 to 1.30 g/ml to achieve maximum resolution of different virus bands. The band of enveloped single nucleocapsids is designated by the arrow 1. Subsequent numbers and arrows designate 2, 3, 4, etc., nucleocapsids per envelope, each with a correspondingly higher density (see relative positions for each band, Fig. 5). For conditions of sample preparation and centrifugation see the text.*



Knudson and Tinsley (8) demonstrated that ^{3}H-labeled NPV of *Spodoptera frugiperda* purified from CCM and banded to equilibrium in sucrose gradients had a mean density of 1.21 to 1.22 g/ml and ranged from 1.18 to 1.25 g/ml. The preparation appeared to be fairly homogeneous. Alkaline-solubilized virus exhibited more heterogeneity in sucrose gradients than the nonoccluded virus, ranging from 1.15 to 1.24 g/ml, but the major bands also occurred primarily at 1.21 to 1.22 g/ml. The heterogeneity of alkali-liberated *Spodoptera* virus is understandable, since the virus also belongs to the class of *M*NPVs. Nevertheless, it is curious that the major component of the alkali-liberated virus preparation (presumably bundles of several nucleocapsids per envelope) is of the same density as the nonoccluded virus from tissue culture (presumably single nucleocapsids per envelope). In explanation, the authors suggested that the nonoccluded virus was a population of single, unbundled virions, and that the alkali treatment of the occluded virus had stripped the envelopes from some of them causing them to band out as unenveloped nucleocapsids. The results of this combined study, using electron microscopy and physical separation correlated with biological activity, confirm that the infectious entity from CCM and infectious hemolymph is primarily in the form of single, loosely enveloped nucleocapsids. The comparisons were made on each of three different sources of two different viruses.

Further, the results suggested that, whereas the nonoccluded form of the *M*NPVs derived from hemolymph and CCM are similar, they are very different from the alkaline-liberated form of the occluded *M*NPVs. From these results, one might expect serological differences among viruses from the three sources, and indeed there are. We have found (manuscript in preparation) that antisera raised against alkali-liberated virus inhibits in vitro plaque formation by homologous virus, but does not interfere with plaque formation by the nonoccluded form. These data suggest the necessity of biochemical, biophysical, and related serological comparisons of virus not only from different sources in the same host, but also from different sources in alternate hosts, to detect any virus modifications and, once detected, to determine the

FIG. 7. *Thin section containing AcMNPV in the process of exocytosis from a TN-368-10 cell. For method of preparation see the text.* ×75,000.

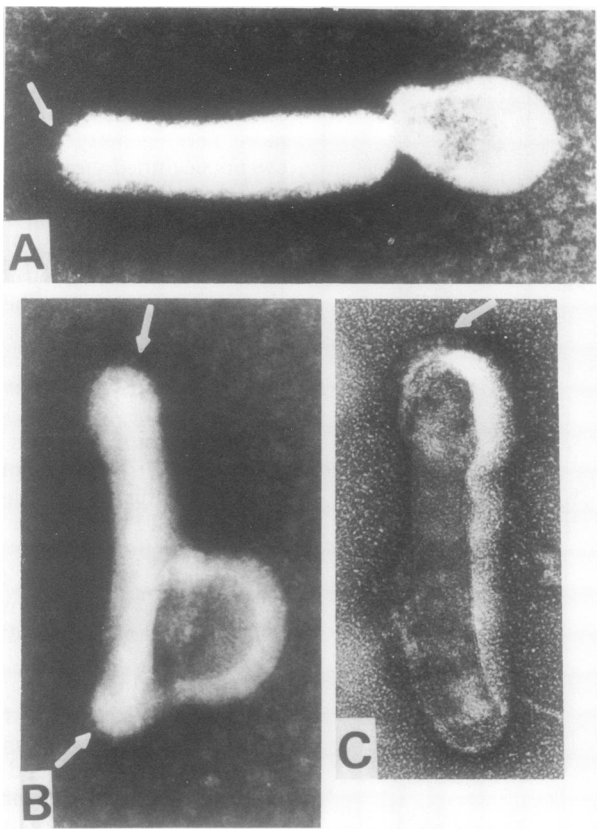


FIG. 8. *Preparation of AcMNPV (12G pellet of CCM) negatively stained with ammonium molybdate (pH 7.2) showing the presence of viral envelope in loose and/or variable association (A and B, respectively) and the presence of surface projections or peplomers on the viral envelope (arrows). ×172,000.*

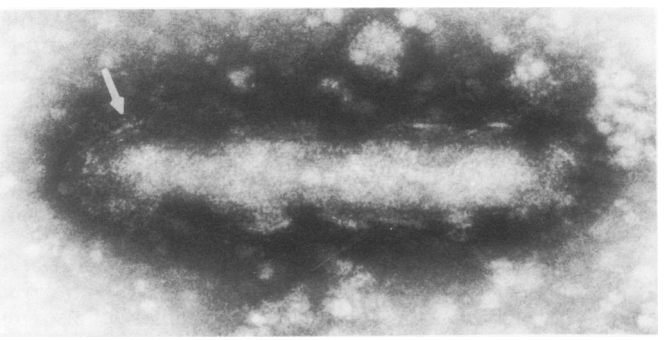


FIG. 9. *Preparation of AcMNPV from hemolymph (12G pellet) negatively stained with ammonium molybdate showing enveloped particle (partially disrupted) with surface projections (arrow). ×186,000.*

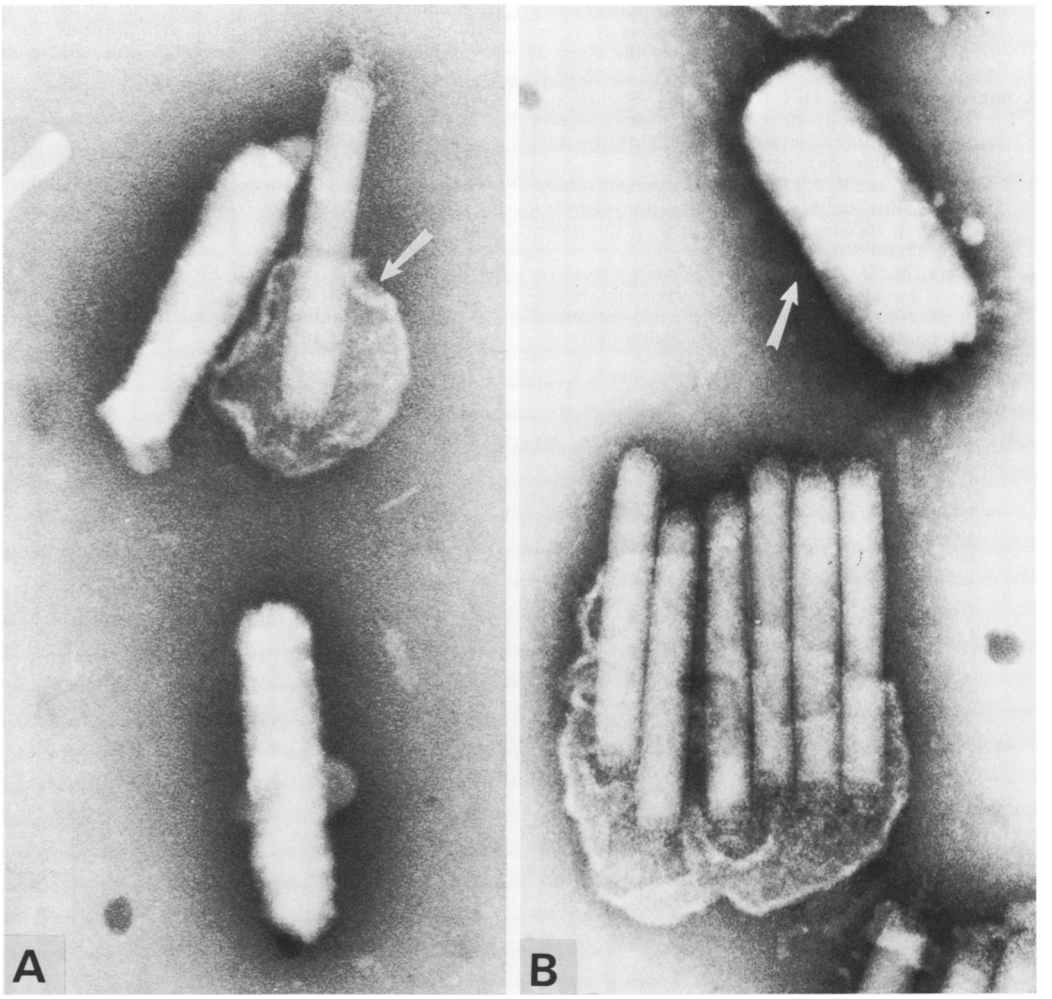


FIG. 10. *Preparation of alkali-liberated and gradient-purified AcMNPV (Fig. 6B) negatively stained with ammonium molybdate. (A) Enveloped single nucleocapsids with envelope intact and partially disrupted (arrow). (B) Many nucleocapsids common to a viral envelope with envelope intact (arrow) and disrupted. ×168,000.*

nature of virus structure relative to these modifications.

ACKNOWLEDGMENTS

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