Mass Purification of Nucleopolyhedrosis Virus Inclusion Bodies in the K-Series Centrifuge

J. P. BREILLATT, J. N. BRANTLEY, H. M. MAZZONE, M. E. MARTIGNONI, J. E. FRANKLIN, AND N. G. ANDERSON

Molecular Anatomy Program and Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830; Northeastern Forest Experiment Station, Hamden, Connecticut 06514; and Pacific Northwest Range and Experiment Station, Corvallis, Oregon 97331

Received for publication 12 January 1972

Nucleopolyhedrosis virus inclusion bodies specific for Hemerocampa pseudotsugata, Neodipion sertifer, Porthetria dispar, and Heliothis zea have been purified by using a continuous-sample-flow-with-isopycnic-banding centrifuge in quantities up to $6 \times 10^{13}$ polyhedral inclusion bodies per day. Continuous-flow methods for $S_p$ type purification have been evolved to deal with mass isolation of bioparticles.

The use of nucleopolyhedrosis viruses for biological control of insects is under study in many laboratories. Maximal use of the agents will require maintenance of microbial contaminants at safe levels and may require removal of the potent insect allergens present in some species. For example, the hairs of the Douglas-fir tussock moth and the gypsy moth caterpillars can cause a severe contact dermatitis in humans. These hairs are present in crude homogenates, and if formed into an aerosol for aerial application, would constitute an obvious danger to mammalian respiratory tracts.

We have investigated the use of large-scale density gradient centrifugation for the purification of the polyhedral inclusion bodies (PIB) of nucleopolyhedrosis viruses. The initial isolation of polyhedrosis virus PIB by density gradient centrifugation was in swinging-bucket rotors (12). While this technique remains unsurpassed for analytical purposes, as a preparative method it is limited in the quantity of product it yields.

The batchwise use of the B-XXIII and the B-XV zonal rotors resulted in the isolation of $3 \times 10^{11}$ pure PIB per run from Douglas-fir tussock moth larvae (Hemerocampa pseudotsugata) (13). We have since isolated PIB specific for the corn earworm (Heliothis zea), the European pine sawfly (Neodiprion sertifer), and the gypsy moth (Porthetria dispar) in similar quantities in the B-XXIX rotor (3). While this represents a considerable gain over the amounts obtainable from the swinging-bucket rotor, the quantities isolated are still short of those required for field use of PIB as insect control agents.

We therefore turned to large-scale continuous-sample-flow-with-isopycnic-banding centrifugation in the K-series system (4). A new rotor, K-X, designed for isolation of micrometer-sized particles has been used successfully to prepare pure PIB in quantities of $6 \times 10^{13}$ per run from the diseased larvae of each of the four insect species previously mentioned. Preliminary results of this work have been reported (7).

Significant advances in the methodology of continuous-sample-flow-with-isopycnic-banding centrifugation have been made in evolving the present technique for PIB isolation from larval homogenates.

MATERIALS AND METHODS

Sample preparation. The larval preparations were: $H. zea$, Biotrol-VHZ, a lyophilized extract containing $3 \times 10^{14}$ PIB/g, obtained from Nutrilite Products, Buena Park, Calif. $N. sertifer$ and $P. dispar$: diseased larvae were homogenized in water and the suspension was passed through cheesecloth. The material was then centrifuged at low speed to remove large contaminants. The resulting aqueous suspensions contained ca. $2 \times 10^6$ and $5 \times 10^5$ PIB/ml, respectively. An alternate preparation from $P. dispar$ was made by disrupting diseased larvae and suspending them in water. The material that settled to the bottom of the container was collected, dried, and ground.

All subsequent procedures were carried out in media containing 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride, 0.00075% dioctyl sodium sulfosuccinate, pH 7.2-7.4 at 25 C (TD buffer). All sucrose concentrations are expressed as percent w/w.

In the cases of $H. zea$, $H. pseudotsugata$, and the dried $P. dispar$, a 5% larval homogenate in 43%
sucrose was prepared in a 1-gallon Waring Blender at top speed for 2 min and then filtered through cheesecloth.

The N. setifer and P. dispar aqueous suspensions were diluted with 66% sucrose to a concentration of 43% sucrose and homogenized in the Blender to ensure dispersion of the PIB.

Centrifugation. An aluminum K-X rotor (J. P. Breillatt, J. N. Brantley, and R. F. Gibson, manuscript in preparation) was operated in a K-series centrifuge with a C-type casing (4) equipped for temperature control of the rotor and fluid flow lines (6). Sample suspensions were held in 20-liter pressure cans (model XX67-000-05 pressure tanks, Millipore Corp., Bedford, Mass.) with the sample flow stream propelled by air pressure. The sample flow rate was regulated by a throttle valve on the outflow line and monitored with a calibrated flow meter or by sample collection.

Gradients were loaded and recovered with the rotor at rest. The gradients were reoriented into the spin configuration by controlled acceleration (4): 0 to 500 rev/min at 2 rev per min per sec, 500 to 2,000 rev/min at 4 rev per min per sec. Sample flow was initiated at 2,000 rev/min, and the rotor was accelerated under full drive air pressure to the final velocity. After exhaustion of sample, the rotor was operated at 35,000 rev/min for the indicated time interval, to band the PIB, and the rotor was decelerated by the reverse of the controlled-acceleration procedure. The gradients were recovered as 100-ml fractions through the virus peaks and as 400-ml fractions elsewhere.

Gradient recovery was monitored at 580 nm by a spectrophotometer equipped with a flow cell and by microscopy inspection of the recovered fractions. The fractions containing pure PIB were diluted 1:1 with TD buffer and centrifuged in a Spinco type 19 rotor or passed through a K-XI rotor (J. P. Breillatt, R. M. VanFrank, R. F. Gibson, and J. N. Brantley, manuscript in preparation). The pellets were pooled and washed once by centrifugation in TD buffer and then resuspended in the same buffer. The less pure fractions from the sides of the PIB peak were carried through the same procedure separately.

Theoretical calculations. Fractional cleanout of particles from the sample flow stream was predicted by equation 29 of Sartory (17):

\[
F = \frac{8\pi \gamma r L}{10^4} \frac{(\rho_p - \rho_m)}{\eta_m (t/f_{min})} S, \tag{1}
\]

where \( F \) is the fractional cleanout of the particle species of interest (p) and is defined as \((P_{\text{final}} - P_{\text{initial}})/P_{\text{initial}}\), \( r \) is the rotor core radius, \( L \) is the effective rotor core length, \( Q \) is the sample flow rate (liters/hour), and \( S \) is the sedimentation coefficient of the particle (expressed in Svedberg units at the sample flow stream temperature and composition).

Since the sedimentation coefficients of large bio-particles are not as commonly known as their diameter and isopycnic density, \( S \) can be replaced in equation 1 by

\[
S = \frac{D_p^2}{18} \left( \frac{\rho_p - \rho_m}{\eta_m (t/f_{min})} \right) 10^{14} \tag{2}
\]

to yield

\[
F = \frac{4\pi \gamma r L D_p (\rho_p - \rho_m) (\text{rev/min})^2}{9 \times 10^4} \frac{1}{\eta_m (t/f_{min})} \frac{Q}{S}, \tag{3}
\]

where \( D_p \) is the diameter of the particle (centimeters), \( \rho_p \) is the density of the solvated particle, \( \rho_m \) and \( \eta_m \) are the density and viscosity of the sample flow stream at operating temperature \( (\eta_m \) in poises), and \( t/f_{min} \) is the fractional coefficient. In the case of the near-spherical PIB, we have set \( t/f_s \) equal to 1 and have neglected the hydration term.

In practice these equations (equations 1 and 3) have been found to be optimistic by a factor of 2 to 3 (16) for possible reasons discussed by Sartory (17). Accordingly, after solving for a flow rate at 100% cleanout, the flow rate used is one-half to one-third that calculated.

Quantitation. PIB concentration of sample and gradient fractions was determined by particle counts in a Petroff-Hausser counting chamber or a Bright-Line hemocytometer using phase-contrast optics. Microorganisms were assayed by using standard plate count agar (Difco) for total bacterial counts and violet red bile agar (Difco) for coliforms (1).

RESULTS

Considerations of purification cost led us initially to seek rotor velocity and sample flow parameters applicable to a large centrifuge design in the 5,000 rev/min range with flow rates of 100 to 200 liters/hr of 5% larval homogenate. As a test system, flow rates for the K-X rotor were calculated (5% homogenate in TD buffer at 25 C) to be 25 and 100 liters/hr at rotor velocities of 5,000 and 10,000 rev/min.

One kilogram of Biotrol-VHZ was homogenized in 18 liters of TD buffer and passed over a 10 to 65% sucrose gradient in the K-X rotor at 24 liters/hr and 5,000 rev/min. Microscopy analysis of the effluent showed removal of most of the PIB but, on recovery of the gradient, the sectors unloaded unevenly due to large amounts of debris in the gradient that clogged the rotor drain lines. Analysis of the debris and the gradient showed this material to band isopycничially in 40% sucrose as a thick gelatinous mass, thereby partially inhibiting PIB transport through the gradient to their equilibrium position at 57% sucrose. In addition, a lipid layer 3 mm thick had formed on the core surface, while a 5-mm layer of dense brown debris had built up on the outer wall of the rotor cavity, thereby displacing the gradient.
These initial results led us to seek conditions selectively to inhibit unwanted particle entry into the gradient. Efficiency of removal of a particle from the sample flow stream is a direct function of its sedimentation coefficient; therefore, through manipulation of the sample stream flow rate and rotor velocity, we can effectively cut off the entry into the gradient of particles with sedimentation coefficients below a given value. By using two rotors in cascade (Fig. 1), the first to remove unwanted particles of high sedimentation coefficient, the second to pass unwanted particles of low sedimentation coefficient, only particles within a preselected sedimentation coefficient interval will be captured in the gradient of the second rotor. The same effect can be achieved by sequentially performing the two separate operations in a single rotor.

The first rotor operates under conditions such that 1% of the particles of interest (p1) are removed from the flow stream. Then particles with sedimentation coefficients 10 times that of p1 will be removed from the stream at 10% efficiency, and those particles with sedimentation coefficients 100 times that of p1 will be completely removed from the sample stream. Thus the effluent from the first centrifugal stage (characterized by relatively low rotor velocity and high sample flow rate) will contain 99% of the desired particles and very few more rapidly sedimenting contaminants. This effluent is passed through the second rotor under conditions of flow and rotor velocity such that 100% of the desired particles remaining in the effluent from the first rotor are pulled into the gradient. Particles whose sedimentation coefficients are one-tenth that of p1 will move into the gradient at 10% efficiency, while the particles leaving the flow stream at 1% cleanout will have sedimentation coefficients one-hundredth that of p1.

One of the most powerful concepts in virus and bioparticle isolation is the S-p separation scheme (2). Following a suggestion by Carl Price (personal communication), we have departed from the usual form of the S-p plot, which employs the sedimentation coefficient of the particles corrected to water at 20 C, and have used the sedimentation coefficient of the particles in the sample medium. This permits a more realistic judgment of the potential resolution of the separation process, primarily by emphasis of the flotation (negative sedimentation coefficient) of particles less dense than the sample medium. When the results of the rotor-velocity-sample-flow-rate manipulations are

\[
\text{RPM : 3,500} \quad \text{Q/3 : 300} \quad \text{I/hr} \\
\begin{array}{ll}
S & F \\
5 \times 10^4 & 1 \% \\
5 \times 10^5 & 10 \% \\
5 \times 10^6 & 100 \% \\
\end{array}
\]

\[
\text{RPM : 35,000} \quad \text{Q/3 : 30} \quad \text{I/hr} \\
\begin{array}{ll}
S & F \\
5 \times 10^4 & 100 \% \\
5 \times 10^5 & 10 \% \\
5 \times 10^6 & 1 \% \\
\end{array}
\]

**Fig. 1.** Two continuous-sample-flow-with-isopycnic-banding rotors arranged in cascade to create a sedimentation coefficient interval between \(5 \times 10^4\) and \(5 \times 10^6\) S in 43% sucrose at 25 C. Particles whose sedimentation coefficients fall in this range will be preferentially collected in the second rotor. Fractional cleanout values (F), sedimentation coefficients (S), and the corrected volumetric flow rate (Q/3) are calculated from equations 1 and 2 for representative PIB.

plotted on the S-p plot (Fig. 2), a sedimentation coefficient interval is observed within which particles enter the gradient, there to be further resolved by isopycnic banding. While this does not constitute a single-step S-p procedure, the end result, with kilogram quantities of material, is practically identical with that of such a procedure. A similar degree of separation efficiency is not currently feasible by other methods.

For the present case, this technique excludes from the final purification gradient all large, dense debris and all lipids and small contami-
nating particles, which together comprise the bulk of the undesirable particles in the larval homogenate.

These considerations require a sample flow rate of 300 liters/hr in the first rotor (Fig. 1 and 2). However, flow rates above 50 to 60 liters/hr are not satisfactorily obtained in the present system under the conditions used. At the maximum flow rate used (48 liters/hr), the 1% cleanout level corrected for nonideal flow conditions in the sample stream is at $7 \times 10^3$ S (PIB of 1.0 μm diameter), and the 100% cleanout level is at $7 \times 10^3$ S (PIB of 10 μm diameter). Due to this lower sample flow rate, more of the PIB population is collected in the first rotor than originally intended in the theoretical design (Fig. 2). However, this departure from the theoretically derived procedure is of little import, since the PIB collected in the first rotor are recovered from the gradient, diluted, and added to the sample flow stream of the second rotor before flow through the second rotor is completed. Indeed it is with the first rotor that one has the greatest freedom to vary the run parameters to selectively avoid more rapidly sedimenting contaminants.

The other source of interfering material in the sample was the substance that banded at about 40% sucrose and inhibited gradient recovery and inclusion body transport within the gradient. It was excluded from the gradient by adding sucrose to the homogenate until the sample density equalled the isopycnic banding density of the interfering particle. This caused the particle to remain in the sample stream and permitted the gradient to be designed solely for high-resolution separation of PIB from bacteria, spores, and other particles of similar size and density present in the larval homogenate.

Because the rate of removal of PIB from the flow stream (and thereby the time-cost relationship of purification of the inclusion bodies) is related to the density and viscosity of the sample flow stream, the density of the sample homogenate was increased just to the level that would exclude the unwanted particles, i.e., 43% sucrose. This maximized the flow rate achievable under conditions that minimized the appearance of this specific contaminant in the gradient; nevertheless, the sedimentation rate of the inclusion bodies was reduced 22-fold. For this technique, a density-creating solute with negligible viscosity might be preferable to sucrose, regarding the rate of particle removal from the flow stream; however, if sucrose were still used for the gradient, at these densities an appreciable cross-diffusion of gradient solute and sample density solute (if different) would occur, creating a complicated situation with respect to particle transport and pressure drop along the sample flow stream.

The increase in sample density and viscosity necessitated an increase in rotor velocity to 25,000 rev/min to maintain 100% cleanout of the inclusion bodies at 25 liters/hr. Thus the K-X rotor, which was to be a test system for a
conceptual large, slow rotor, now approaches the optimal-design rotor for this separation due to the presence in the larval homogenate of an interfering particle. It is possible that this interfering substance occurring in *H. zea* preparations is not present in all species, thus permitting the use of more efficient conditions.

To evaluate the gradient requirements, 250 g of Biotrol-VHZ was homogenized in 4.75 liters of 43% sucrose and passed over a 47 to 65% sucrose gradient in a K-X rotor at 15,000 rev/min, 11 liters/hr, 25 C. The interfering material did not enter the gradient. The PIB banded as a single peak. However, on microscopy inspection of the peak fractions, numerous dead yeast cells and other material were observed. The peak fractions were pooled, pelleted, and then isopycnilly banded in a B-XXIX rotor containing a 45 to 62% sucrose gradient. Four peaks were resolved, with dead yeast cells banding at 60 and 58.2% sucrose, the inclusion bodies at 56.8% sucrose, and miscellaneous debris below 50% sucrose.

A gradient was designed to exclude the contaminating particles based on these data and the following rationale. The banding positions of unwanted particles (58.2 and 50% sucrose) should be far from the isopycnic position of the inclusion bodies (56.8% sucrose). The PIB banding zone should be a broad flat region in the gradient to allow maximum capacity for PIB banding without formation of a thickly packed zone that would block passage of denser, but more slowly sedimenting, contaminants. A stacked gradient was chosen for ease of operation and because it closely approached the ideal gradient; however, once the gradient is formed in the rotor, diffusion rapidly obliterates any sharp steps (Fig. 3).

The gradient was tested by passing a 25-liter preparation from *N. sertifer* (2 × 10^14 PIB) through the rotor at 25 liters/hr and 30,000 rev/min. The first pass through the low-velocity rotor was neglected in this case because of the prior removal of large debris during sample preparation. PIB cleavage efficiencies of 85% per pass through the rotor were obtained with 97% cumulative recovery of PIB by recycling the effluent through the rotor (14). The PIB were well resolved from debris and contaminants, as shown by the gradient profile (Fig. 3) and by microscopy analysis of the recovered peak fractions which had the macroscopic appearance of a creamy white slurry. In subsequent runs, quantities up to 6 × 10^13 PIB have been processed in a single rotor.

To test the entire system, 1.2 kg (wet weight) of diseased *H. pseudotsugata* larvae was prepared and passed through a K-X rotor operating at 3,500 rev/min with flow rates of 42 to 48 liters/hr. The rotor contained a step gradient of 2 liters of 60% sucrose and 4.7 liters of 47% sucrose. After termination of flow the rotor was operated at 30,000 rev/min for 15 min to permit the large particles to reach equilibrium. The fractions containing PIB were recovered from the rotor, pooled, diluted with TD buffer to 43% sucrose, and added back to the effluent. This effluent was then passed through a K-X rotor at 24 liters/hr, 30,000 rev/min, 22 C, over the derived gradient (Fig. 4A). After exhaustion of the sample, the rotor was operated at 35,000 rev/min for 70 min to bring the larger particles to their isopycnic density. These field-collected larvae suffered from a mixed infection, as shown by the cytoplasmic polyhedrosis inclusion body peak at 1 liter gradient volume. Microbiological assay of the recovered fractions showed that certain of the microorganisms banded isopycnilly in the same density range as the PIB (Fig. 4B).

When the dried *P. dispar* larvae preparation was processed by a two-step procedure in the K-X rotor (in the same manner as the *H. pseudotsugata*), upon recovery of the gradient from the second rotor all fractions contained visible debris. This was traced to dense fragments insufficiently solvated to stick to the wall of the first rotor. When the gradient was recovered from the first rotor (at rest), the debris washed off the wall into the various fractions; when the fractions containing PIB were added back to the sample for the second rotor, the process repeated itself. The PIB from that particular run were pooled and banded in a B-XV rotor and then dynamically unloaded to remove the contamination. Subsequent *P. dispar*
samples were prepared as the aqueous suspension. This solvated the debris to the extent that it adhered to the K-X rotor wall during the unloading procedure (Fig. 5A) and removed the need for the centrifugation step in the B-XV rotor.

As seen in Fig. 4B for *H. pseudotsugata* and in Fig. 5B for *P. dispar*, microorganisms are found in higher concentrations in the PIB banding density range (possibly spores whose sedimentation was retarded by the tightly packed PIB in the zone) and also at the periphery of the rotor (gradient volume of 0 to 1 liter). By analogy to the debris problem, one would expect contamination of all fractions by pelleted bacteria or spores to the extent that they were washed off the wall during static gradient recovery. Dynamic gradient unloading could avoid this problem, but a high-resolution method to unload dynamically a continuous-sample-flow K-series rotor has not yet been devised.

While satisfactory results were obtained with PIB isolations from *N. sertifer*, *P. dispar*, and *H. pseudotsugata* larvae in the amounts available, a large-scale isolation from a debris-laden sample was necessary to investigate the capacity of the cascaded K-X rotor system. Two kilograms of Biotrol-VHZ (6 × 10^{13} PIB) was processed through two K-X rotors in cascade: the first operated at 3,500 rev/min with a sample flow rate of 42 to 48 liters/hr, the second operated at 35,000 rev/min with a sample flow rate of 20 to 24 liters/hr. The sample was passed through the second rotor twice, with approximately 80% cleanup of PIB per pass through this rotor as judged by hemocytometer counts on the sample and effluents. During this run, lipid-containing material accumulated in the inflow lines of the rotor and was prevented from entering the rotor cavity by the density difference of the material and its suspending medium, 43% sucrose. In this case the \( \rho_p - \rho_m \) term (equation 2) produced a negative sedimentation coefficient (a flotation term) for the lipid-containing particles, which accumulated in the lines and effectively blocked the sample flow. The lipid block was periodically removed by back-flushing the lines. This recovered material was extracted with methanol-chloroform and yielded a dense precipitate, easily sedimentable in 45% sucrose, plus a yellow solution. A lipid solvent that does not inactivate the PIB or other preliminary steps to remove the lipid would solve this problem, since a suitable modification of the rotor is not readily apparent.

Fig. 4. Isolation of PIB from *H. pseudotsugata* larvae by using a sedimentation coefficient interval created by sequential use of a K-X rotor. (A) Gradient profile from second rotor; ●, recovered gradient. (B) Bacteria distribution in fractions recovered from second rotor.

![Graph showing sedimentation coefficient profile](image)

Fig. 5. Isolation of PIB from *P. dispar* by using a sedimentation coefficient interval created by sequential use of a K-X rotor. (A) Gradient profile from second rotor. —, Initial gradient; ●, recovered gradient. (B) Bacteria distribution in fractions recovered from second rotor.

![Graph showing sedimentation coefficient profile](image)
Microbiological assay of the pooled PIB preparations (Table 1) yielded the following conclusions. Significant reductions in bacterial counts were achieved only with *N. sertifer* which had been precleaned and with *H. zea* which was produced in a controlled insectary, suggesting that the strains of bacteria that band in the same density range as the PIB may be species-specific, removed by the precleaning step, or absent from the insectary environment.

The infectivity of the inclusion bodies in their respective hosts was not changed by the centrifugal purification process.

**DISCUSSION**

The inclusion bodies purified by this method are free from insect components and debris on microscopy inspection. The significance of this fact for studies on inclusion body protein and virus components is evident. The significance of the fact for use of the inclusion bodies as a biological control agent is questioned by several studies which show that crude and semipurified preparations of nucleopolyhedrosis virus inclusion bodies from *H. zea* and *Trichoplusia ni* are nontoxic and nonpathogenic in the guinea pig and only mildly allergenic in this animal (9, 10, 15). A common finding in the two studies on *H. zea* was that a guinea pig from the inhalation test group died from pneumonia after exposure to the preparation, which seems to preclude allergenicity but may indicate a primary reaction to the agent. The possible significance of this occurrence may be found in considering *H. pseudotsugata* larvae, whose airborne hairs cause a mild to severe contact dermatitis to loggers and farmers working in the woods during an outbreak of the insect. In studies with rabbits, crude preparations of *H. pseudotsugata* were nonallergenic but caused a minor primary skin irritation (18). This raises the question of whether guinea pigs may have an individual primary response to these agents, as do humans, and emphasizes that animal reaction to a larval preparation must be considered separately for each insect species.

The reaction of animals to *N. sertifer* inclusion bodies purified by this method and to the crude starting material is being assayed by an independent testing company for the Northeastern Forest Experimental Station of the Forest Service (U.S. Department of Agriculture). To date no pathogenicity, toxicity, or allergenicity has been found in a number of standard test procedures (F. B. Lewis, personal communication).

However, the responses of large sectors of the human population repeatedly exposed to large quantities of airborne insect dusts are not known in detail. In addition to dermatitis, cases of conjunctivitis and asthma have been reported in human beings after exposure to body parts of various arthropods, even as dusts of dead insects. Furthermore, the intended or accidental contamination of food crops with products containing insect fragments may lead to an excess of the tolerances for the ratio of insect origin set by the Federal Food, Drug, and Cosmetic Act. The origin of insect fragment contamination can be traced by means of refined methods of sanitation-analytical entomology (11).

Even though the medical and legal implications of large-scale field applications of crude preparations have not yet been fully evaluated, it would seem cautious and advisable to strive to reduce the insecticidally inactive bulk (mostly insect debris) of nucleopolyhedrosis virus preparations.

The reduction of bacteria in the PIB preparations purified by the present technique was not as great as that accomplished by the two-step batch procedure in the B-series rotors (13). This may be due to several factors previously discussed herein, primarily that of static versus dynamic gradient recovery. The counts reported for coliform bacteria were those present on the violet red bile agar; however, we suspect that these may not truly represent enteric bacilli because of the gray

### Table 1. Bacterial contamination of homogenate and purified PIB

| Species                | Homogenate | Pooled peak fractions |
|------------------------|------------|-----------------------|
|                        | Total aerobic bacteria per 10^8 PIB | Coliform bacteria per 10^8 PIB | Total aerobic bacteria per 10^8 PIB | Coliform bacteria per 10^8 PIB |
| *Hemerocampa pseudotsugata* | 3.7 x 10^7 | 9.1 x 10^6 | 1.7 x 10^7 | 7 x 10^6 |
| *Neodiprion sertifer*    | 4.0 x 10^7 | 7.7 x 10^6 | 1.2 x 10^7 | 5.1 x 10^6 |
| *Porthetria dispar*      | 1.5 x 10^7 | 5.9 x 10^6 | 7.1 x 10^7 | 2.1 x 10^7 |
| *Heliothis zea*          |            |           |            |            |
coloration of the colonies. Several unsuccessful attempts were made to identify the predominant species. During the preparation of this manuscript, Cline et al. (8) have reported that debris and bacterial contamination of zonally purified PIB can be markedly reduced by allowing bacterial digestion of the larval homogenate starting sample, as used by Bergold (5). This is followed by an overgrowth of a bacillus, thus necessitating resolution of the PIB from only a single microorganism.

This appears to be the method of choice for future PIB purification schemes employing the zonal centrifuge, because it also digests those substances which reduced the efficiency of the continuous-flow centrifugation step.

High-resolution separations in the zonal centrifuge are usually synonymous with narrow, low-capacity zones. We have attempted to create conditions in which very large amounts of product (PIB) can be separated, with relatively high resolution, from contaminants (bacteria and spores) differing only slightly in density. This has resulted in unusually wide, thickly packed peaks in shallow density gradients. To permit the use of these gradients, it has been necessary to exclude the bulk of the contaminants from the gradient through manipulation of the rotor velocity and sample density and flow rate.

The concepts of a sedimentation coefficient interval and sample density inhibition of particle transport into the gradient are generally applicable to all bioparticle separations in continuous-sample-flow rotors. They greatly increase the capacity and efficiency of the K-series centrifuge and are particularly useful for preparations containing large volumes of unwanted particles. Their use should permit employing much cruder preparations as samples than those currently used, since pre-cleanup procedures on volumes compatible with use of the K-series centrifuges are unwieldy.

ACKNOWLEDGMENTS

We express our appreciation to John Carnegie for his interest and assistance with the purification of the *H. pseudotsugata* PIB, to Carl S. Rehnborg for providing samples of Biotrol-VHZ, and to Franklin B. Lewis for his continued interest and preliminary information on test results.

The Molecular Anatomy (MAN) Program is supported by the National Cancer Institute, the National Institute of General Medical Sciences, the National Institute of Allergy and Infectious Diseases, and the U.S. Atomic Energy Commission. Oak Ridge National Laboratory is operated by Union Carbide Corporation Nuclear Division for the U.S. Atomic Energy Commission.

LITERATURE CITED

1. American Public Health Association. 1967. Standard methods for examination of dairy products, 12th ed. American Public Health Association, Inc., New York.

2. Anderson, N. G., W. W. Harris, A. A. Barber, C. T. Rankin, Jr., and E. L. Candler. 1966. Separation of subcellular components and viruses by combined rate- and isopycnic-zonal centrifugation. Nat. Cancer Inst. Monogr. 21:253-283.

3. Anderson, N. G., C. E. Nunley, and C. T. Rankin, Jr. 1969. Analytical techniques for cell fractions. XV. Rotor B-XXIX—a new high-resolution zonal centrifuge rotor for virus isolation and cell fractionation. Anal. Biochem. 31:255-271.

4. Anderson, N. G., D. A. Waters, C. E. Nunley, R. F. Gibson, R. R. Schilling, E. C. Denny, G. B. Cline, E. F. Babelay, and T. E. Perardi. 1969. K-series centrifuges. I. Development of the K-II continuous-sample-flow-with-banding centrifuge system for vaccine purification. J. Biochem. 32:460-494.

5. Bergold, G. H. 1953. Insect viruses, p. 91-139. In K. M. Smith and M. A. Lauffer (ed.), Advances in virus research, vol. 1, Academic Press Inc., New York.

6. Brantly, J. N., D. D. Willis, J. P. Breillatt, R. F. Gibson, L. C. Patrick, and N. G. Anderson. 1970. K-series centrifuges. IV. Temperature control. Anal. Biochem. 36:434-442.

7. Breillatt, J. P., M. Martignoni, and N. G. Anderson. 1969. Virus isolation for large-scale insect destruction. Biophys. J. 9:2-262.

8. Cline, G. B., E. Ryel, C. M. Igoffo, M. Shapiro, and W. Stranehe. 1970. Zonal purification studies of the nucleopolyhedrosis virus of the cotton bollworm *Heliothis zea* (Boddie). Proc. IVth Int. Colloq. Insect Pathol., 25-28 August 1970, College Park, Md.

9. Heimpel, A. M. 1966. Exposure of white mice and guinea pigs to the nuclear-polyhedrosis virus of the cabbage looper, *Trichoplusia ni*. J. Invertebr. Pathol. 8:98-102.

10. Igoffo, C. M., and A. M. Heimpel. 1965. The nuclear-polyhedrosis virus of *Heliothis zea* (Boddie) and *Heliothis virescens* (Fabricius). I. Toxicity-pathogenicity of virus to white mice and guinea pigs. J. Invertebr. Pathol. 7:239-340.

11. Kurtz, O. L., and K. L. Harris. 1963. Micro-analytical entomology for food sanitation control. Association of Official Agricultural Chemists, Washington, D.C.

12. Martignoni, M. E. 1967. Separation of two types of viral inclusion bodies by isopycnic centrifugation. J. Virol. 1:646-647.

13. Martignoni, M. E., J. P. Breillatt, and N. G. Anderson. 1968. Mass purification of polyhedral inclusion bodies by isopycnic banding in zonal rotors. J. Invertebr. Pathol. 11:507-510.

14. Mazzone, H. M., J. P. Breillatt, and N. G. Anderson. 1970. Zonal rotor purification and properties of a nuclear polyhedrosis virus of the European pine sawfly (*Neodiprion sertifer*, Geoffroy). Proc. IVth Int. Colloq. Insect Pathol., 25-28 August 1970, College Park, Md.

15. Meincke, C. F., W. C. McLane, and C. S. Rehnborg. 1970. Inhalation and dermal allergenicity studies of a nuclear-polyhedrosis virus of *Heliothis zea* in guinea pigs. J. Invertebr. Pathol. 15:207-210.

16. Perardi, T. E., and N. G. Anderson. 1970. K-series centrifuges. III. Effect of core taper on particle capture efficiency. Anal. Biochem. 34:112-122.

17. Sartory, W. K. 1970. Fractional cleanup in a continuous-flow centrifuge. Separ. Sci. 5:137-143.

18. Tucker, R. K. 1970. Handbook of toxicity of pesticides to wildlife. Denver Wildlife Research Center Publ. no. 84. U.S. Government Printing Office no. 853-932, p. 88.