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Characterization of Nuclear Polyhedrosis Virus DNAs

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The nuclear polyhedrosis virus DNAs characterized and compared in this study consist of the singly-enveloped nucleocapsids (SNPV) of *Trichoplusia ni* and the bundles of nucleocapsids common to a single envelope (MNPV) from *Spodoptera frugiperda* and *Rachiplusia ou*. The SNPV and MNPV DNAs are very similar in hydrodynamic properties and molecular weights. In addition, the NPV DNAs are similar in size to those extracted from the granulosis viruses that infect *T. ni* and *S. frugiperda*. As isolated from purified virus or directly from occluded virus, the nuclear polyhedrosis virus DNAs consist of a mixture of about 20 to 30% double-stranded covalently closed molecules and approximately 60% relaxed circles, with less than 10% in linear duplex form. The molecular weights of all nuclear polyhedrosis virus DNAs as compared in this study are slightly smaller than those of T4 bacteriophage DNA and perhaps slightly smaller than those of the granulosis virus DNAs. The best estimates of these molecular weights by neutral sucrose sedimentation for the nuclear polyhedrosis viruses range from 90 to 100 $\times$ 10^6 relative to a size of 108 $\times$ 10^6 for T4 DNA. The base compositions of the nuclear polyhedrosis viruses that infect *T. ni* and *S. frugiperda* are compared with the respective insect host DNAs.

Recent studies on the physical and chemical properties of granulosis virus DNAs of *Trichoplusia ni* and *Spodoptera frugiperda* have shown that the DNA genomes liberated from these two viruses are very similar in hydrodynamic properties and size, but differ from each other in base composition. From both viruses, the DNAs were shown to be liberated in the form of double-stranded, covalently closed molecules that could take the form of a superhelix in low-ionic-strength solutions, relaxed circles, and a small percentage (10% or less) of double-stranded, linear molecules. As studied herein, the hydrodynamic and sedimentation properties of the nuclear polyhedrosis virus of *Rachiplusia ou* (bundles of nucleocapsids common to a single envelope will be designated with the letter M, i.e., RMNPV), the singly enveloped nucleocapsids of the nuclear polyhedrosis virus (SNPV) of *T. ni* (TnSNPV), and the MNPV of *S. frugiperda* (SfMNPV) show striking structural and conformational similarities, both with each other and to those properties of the granulosis virus DNAs previously reported (19, 20). In addition, this paper descriptively completes a standardization of the methods using hydrodynamic technique for the further characterization and use of these viral DNAs and attempts to lay the foundation for more definitive studies, especially those studies directed toward characterizing the genetic relatedness of the viral genomes as well as the fate and function of insect virus DNA during the invasion, replication, and maturation processes in vivo and in vitro. Also, these studies provide a basis for a more precise classification on a biochemical and molecular basis for comparisons with and among other occluded insect viruses.

The results of earlier studies that have been reported on the isolated nucleic acids of nuclear polyhedrosis (NPV) and granulosis virus (GV) DNAs have been summarized and reviewed by Summers and Anderson (19, 20). Recently, Kok et al. (11; I. P. Kok, A. V. Chistyakova, A. P. Gudz-Gorban, and A. P. Solomko, Abstr. Int. Cong. Entomol., 13th, Moscow, p. 127, 1968) in their review and study on purified insect virus DNAs have provided additional evidence that DNA isolated from *Bombyx mori* NPV could be observed in electron microscope preparations to have sizes with an upper range of approximately 60 μm. These experiments have now taken on additional importance because of the speculation that there is subgenomic infectivity associ-

ated with purified virus and DNA preparations (6, 11, 25).

MATERIALS AND METHODS

Purification of virus DNA. DNA was isolated directly from occluded virus (inclusion bodies or capsules) or from purified enveloped virus. Virus was released from inclusion bodies after dilute alkaline saline treatment with 0.07 M Na_2CO_3 plus 0.05 M NaCl (pH 10.8) for 2 h and subsequently purified by rate-zonal sedimentation in 10 to 40% sucrose gradients (19, 21). Each NPV and GV preparation was observed embedded in thin sections by electron microscopy to confirm the structural properties of the virus. Enveloped virus was further purified by banding the virus recovered from sucrose gradients on preformed CsCl gradients (density range from 1.1 to 1.4 g/ml). The recovered virus could be treated with DNAse I and again purified as described.

DNA was isolated from the purified virus preparation by two methods; the method of Gafford and Randall (9) was used except that after the chloroform-*n*-butanol extraction, the DNA was not ethanol precipitated, but was dialyzed against 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) at 4 C. The DNA was further deproteinized with Pronase (self-digested for 2 h at 37 C, 1 mg/ml; treatment of the DNA was for 2 h at 37 C with 200 μg of Pronase per ml per 100 μg of DNA per ml). Observations of sedimentation profiles after sedimentation in neutral or alkaline sucrose gradients did not show significant differences after gentle extraction of the preparations with neutralized phenol. DNA was stored in 1× SSC at concentrations of 30 to 100 μg/ml in capped containers at 4 C and over chloroform. The second and usual procedure giving the best yield of DNA was treatment of the purified virus with 4% (wt/vol) sodium lauryl sarcosinate (Sigma Chemical Co.) in 1× SSC plus 10 mM EDTA, after which it was heated in a water bath at 60 C for 30 min. Ethidium bromide was added to the virus sample to a final concentration of 200 μg/ml. This was layered directly on an ethidium bromide-CsCl gradient (16) and centrifuged as described later. This allowed the separation of covalently closed DNA from the linear and relaxed circular forms. Each band was isolated from the gradient, extracted three times with isoamyl alcohol, and dialyzed extensively against 1× SSC before use or storage or both.

DNA could be extracted directly from the occluded virus. After adding the dilute alkaline saline (0.07 M NaCl plus 0.03 M Na_2CO_3, pH 10.8) to purified occluded virus, the preparation was made 4% with respect to sodium lauryl sarcosinate in 1× SSC plus 10 mM EDTA and heated at 60 C for 30 min. Ethidium bromide (200 μg/ml) was added to the lysed, occluded virus preparation, and the sample was immediately layered on ethidium bromide-CsCl gradients and centrifuged as stated above.

Previously, occluded virus had been routinely lyophilized and stored at 4 C. Occluded virus yielded enveloped virus when suspended in dilute alkaline saline, but the recovery of covalently closed DNA is not possible (19, 20). Therefore, lyophilized occluded virus was not employed in this study.

Purification of host DNA. DNA was isolated from pre-pupae or pupae essentially by the method of Laird and McCarthy (12) that involved preliminary purification of the nuclei by differential centrifugation, lysis with 2.0% (wt/vol) sodium dodecyl sulfate in the presence of 0.1 M EDTA, and extraction with neutralized phenol. Again, the DNA was not precipitated, but rather was dialyzed against 0.1× SSC at 4 C. The dialyzed preparation was treated with 50 μg of ribonuclease A and 200 μg of alpha-amylase per ml as described by Laird and McCarthy, re-extracted with phenol, and extensively dialyzed.

Cesium chloride equilibrium centrifugation. Cesium chloride gradients containing ethidium bromide (Calbiochem) were prepared and run essentially according to Radloff, Bauer, and Vinograd (17) with the exception of the addition of 10 mM EDTA. The gradients for the preparative ultracentrifuge had a total volume of 5.0 ml, a mean density of 1.55 g/ml even when preformed, and contained ethidium bromide at a concentration of 200 μg/ml. After adding ethidium bromide to a final concentration of 200 μg/ml to the DNA, the sample was immediately layered on the gradients and centrifuged. Centrifugation was usually for 24 to 48 h at 40,000 rpm and 20 C in an SW65 rotor and L265B preparative ultracentrifuge. After centrifugation, samples were visualized and removed from the tubes while illuminated with a UV lamp.

Neutral CsCl gradients for bouyant density measurements were made both in an analytical ultracentrifuge essentially according to Mandel, Schildkraut, and Marmur (14) (centrifugation conducted at 44,770 rpm for 20 h and 25 C in the analytical ultracentrifuge) and in a preparative ultracentrifuge (24 h at 40,000 rpm and 23 C by using a SW65 rotor and preformed gradients ranging from 1.6 to 1.85 g/ml) by an analogous method, except that T7 bacteriophage DNA and two microbial DNA standards were employed (repurified *Clostridium perfringens* and *Micrococcus lysodeikticus* DNAs from Worthington Biochemical Corp.) in the preparative runs to calibrate the gradients. Optical-grade cesium chloride (Schwarz-Mann, Div. of Becton, Dickinson & Co.) was usually made up in 0.02 M Tris-hydrochloride, pH 8.5, and approximately 2 to 3 μg of insect virus DNA or standard was added to the solution for reference and calibration of the gradients. Density values and base compositions of the standard DNAs were taken from Szybalski (22).

The gradients were fractionated through an ISCO model D fractionator, with a UA-2 UV analyzer system set at maximum sensitivity. The UA-2 output was connected to a Honeywell 10-inch strip chart recorder to allow more accurate measurements of distances and better correlation with the refractive index of the positions of the peaks. All measurements in this paper were made from the 10-inch chart recordings, but the profiles shown in this paper are tracings directly from the output of the smaller ISCO UA-2 chart recorder.

Thermal denaturation. Thermal denaturation curves (T_m) were made in 0.1× SSC, and the midpoints were evaluated essentially according to Mandel

and Marmur (13) in a Gilford model 2400 spectrophotometer by using T7 bacteriophage DNA as a standard. T7 DNA has a guanine plus cytosine (G+C) content of 50% and therefore a T_m of 74.4 C (22). The T_m determinations that were made at least three times on each sample and that were consistent within 1 degree when normalized to the standard yielded the most reliable base composition data. The DNAs used for the T_m studies were phenol extracted, purified on CsCl gradients, and exhibited hyperchromicities of about 30%.

Neutral sucrose gradients. Sucrose gradients (5.0 ml) were made with an ISCO model 570 gradient former and centrifuged for the designated times in an SW65 rotor at 10 C and 40,000, 50,000 or 62,500 rpm. For molecular weight estimations derived from sedimentation, 5 to 20% (wt/vol) gradients were made according to Burgi and Hershey (5) in 0.1 M NaCl plus 0.05 M sodium phosphate buffer, pH 6.8. Except for the molecular weight estimates, 5 to 30% gradients were used rather than the standard 5 to 20% gradients. The self-sharpening of the peaks did not appreciably affect the relative sedimentation rates in the middle two-thirds of the gradient. Usually, centrifugation was conducted at 62,500 rpm and 10 C in an SW65 rotor, rather than at lower speeds in an SW39 or SW50 rotors. Although the relative sedimentation rates of T7 and T4 DNA did change significantly as a result of rotor speed, the rates relative to linear forms of insect virus DNAs were similarly consistent. Gradients were fractionated by ISCO equipment as described above for the CsCl gradients. This allowed bands containing 0.5 to 2.0 μg of DNA to be significantly detected. At these low concentrations, sedimentation of bacteriophage T7 and T4 DNA relative to each other was empirically shown to be independent of concentration and proportional to the time of centrifugation. The revised sedimentation coefficients, molecular weights, and formula for calculating these values were used as given by Freifelder (8). Molecular weight estimates are given by using the lower and upper size range reported for T4 DNA, 108 and 130 $\times$ 10^6, respectively.

Neutral low ionic strength sucrose gradients. Some sedimentation studies were conducted in neutral sucrose gradients made up in buffers of different ionic strength (1.0 to 0.001) by using Schwarz-Mann density gradient grade sucrose to complement the results of alkaline sucrose gradients, because they allow the three forms of the DNA to be visualized in native rather than their denatured form. Usually 5 to 30% or 5 to 20% sucrose gradients made up in dilutions of 1$\times$ SSC were centrifuged at 62,500 rpm and 4 C for 60 min in an SW65 rotor. In these gradients, sedimentation rates were very concentration dependent, although all bands (fastest were covalently-closed circles with superhelix conformation, second and major were nicked circles, and third of slowest were linear molecules) were clearly resolved.

The use of the Tris-buffer system described by Bode and MacHattie (3) did not allow for consistent and reproducible sedimentation studies on covalently closed DNA. For reasons not presently characterized, covalently closed DNA was not easily or consistently recovered in the absence of divalent ion chelating agents. The loss of this species was much greater than normally found for the degradation of DNA in the absence of DNAse inhibitors.

Alkaline sucrose gradients. Routinely, 2 to 40% (wt/vol) sucrose gradients were prepared and used immediately before each experiment in 1.0 M NaCl plus 0.3 M NaOH plus 10 mM EDTA (7). The sucrose gradients exhibited a pH gradient ranging from 13.0 near the top to 12.5 at the bottom. The steeper gradients were used rather than the usual 5 to 20% gradients because this allowed both the very fast sedimenting peak (denatured, covalently closed) and the unresolved slower sedimenting pair of peaks to be observed on the same gradient. In addition, the steeper gradient allowed an improved and significantly measurable resolution of the two slower sedimenting bands (denatured linear and circular DNA) from each other and from the T4 DNA standard. Sample volumes, usually 10 to 50 μl but not greater than 0.1 ml, were carefully layered on top of the gradient and allowed to sit for 10 to 15 min at room temperature before centrifugation. If larger volumes were used, enough 1.0 M NaOH was added to the sample to give a final concentration of 0.3 M NaOH. Centrifugation was carried out at 62,500 rpm and 20 C for 20 to 60 min as designated.

RESULTS

Ethidium bromide-cesium chloride centrifugation. Without exception, the NPV DNAs exhibited identical ethidium bromide-cesium chloride equilibrium banding profiles (Fig. 1) as those previously shown for the granulosis viruses of *T. ni* and *S. frugiperda* (19). If the virus preparation was lysed directly on top of the gradient or centrifuged without gentle phenol extraction, a very faint band was observed intermediate to the covalently closed or heavy (1.58 g/ml) band and the linear and relaxed circles, or light (1.54 g/ml) band of DNA. Because this latter technique gave the highest yield of DNA from a virus preparation, most of the subsequent studies were conducted with DNA recovered from virus lysed directly on top of these gradients. The yield of covalently closed DNA from NPV preparations was very similar to that obtained from GV reported previously (19) and was routinely 25 to 30% of the total DNA recovered.

Neutral sucrose gradient centrifugation. Figure 2 shows sedimentation scanning profiles for the NPV DNAs utilized in this study as compared with those of the previously characterized GV DNAs of *T. ni* and *S. frugiperda*. These are all shown with special reference to two bacteriophage DNA standards, T7 (32*S*) and T4 (62*S*) DNA with relative sedimentation normalized to that of T4 DNA. It was of concern in these studies to investigate the validity or

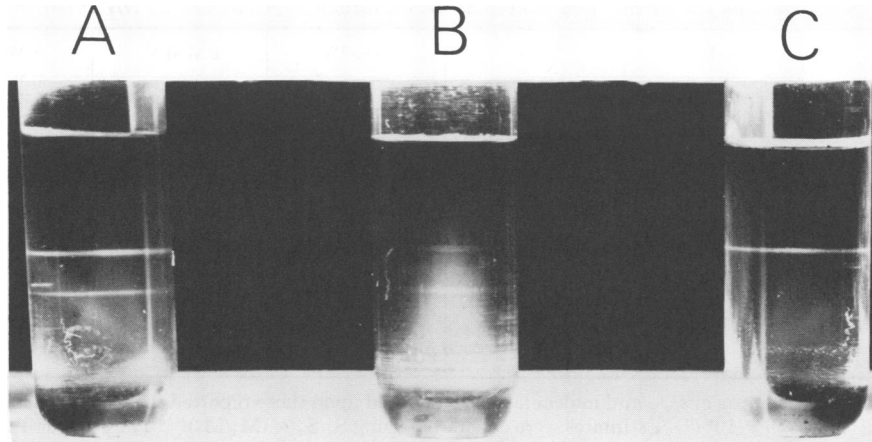

FIG. 1. *Equilibrium banding of SfMNPV (A) and RMNPV (B) DNA in ethidium bromide-CsCl gradients relative to T4 DNA (C). TnSNPV, not shown, was identical to A and B. Centrifugation was carried out at 20 C and 40,000 rpm for 24 h on preformed CsCl gradients (density range 1.4 to 1.65 g/ml) by using an SW65 rotor. Gradients contained 200 μg of ethidium bromide per ml and were prepared with 1× SSC and 10 mM EDTA rather than the Tris-buffer system of Radloff, Bauer, and Vinograd (15). Purified virus in 1× SSC was made 4% (wt/vol) with respect to sodium lauryl sarcosinate to a final volume of 0.5 ml and heated at 60 C for 30 min. Ethidium bromide was added to the lysed virus preparation to a final concentration of 200 μg/ml and immediately centrifuged. Approximately 50 μg of DNA is observed in each of gradients A and B. Tubes were illuminated with a UV lamp and photographed on Kodachrome II film.*

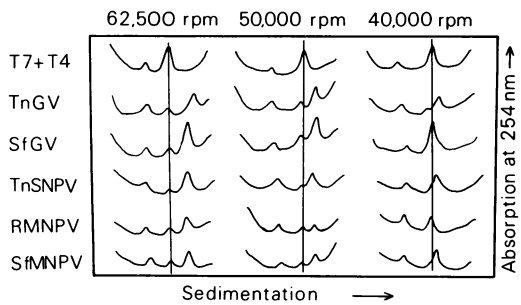

FIG. 2. *Sedimentation in neutral sucrose gradients for an estimate of molecular weights relative to T4 DNA. Gradients were 5 to 20% (wt/vol) made up in 50 mM sodium phosphate buffer, pH 6.8, and 100 mM NaCl. Centrifugation was conducted at 10 C and the speeds shown. Centrifugation times were 70, 105, and 135 min at 62,500, 50,000 and 40,000 rpm, respectively. Peaks of DNA correspond to 2 to 6 μg of DNA (total) loaded on each gradient. Phage DNA standards and sedimentation coefficients are considered as 32 and 62S for T7 and T4 DNAs, respectively. T7 DNA was added (T4 DNA was not added) to each of the insect virus DNA preparations for an internal standard to mark relative rates of sedimentation and to determine the validity of parallel and co-sedimentation with T7 and T4 (T7 is the slowest sedimenting band observed in all gradients shown).*

accuracy of the molecular weight estimates determined by preparative ultracentrifugation because those determinations are made by using well-characterized standards and sedimentation in sucrose gradients and are based upon certain assumptions. Also, because previous centrifugations had been conducted only at 62,500 rpm, it was therefore of interest to see whether or not there was variability in sedimentation behavior dependent upon rotor speed. The sedimentation behavior of the circular species of DNA can vary significantly as a result of rotor speed, but that of the linear duplex does not (Fig. 2, Table 1). In some cases, at a lower rotor speed of 40,000 rpm, the circular form has a tendency to co-sediment, or nearly co-sediment, with the linear duplex. This sometimes resulted in the linear species not being accurately detected and measured at lower rotor speeds where the circular species was present in the higher concentration so as to mask the presence of the linear form.

Table 1 summarizes the estimated molecular weights for the linear forms of insect virus DNAs used in this study. Because the reported estimates of molecular weight for T4 DNA are quite variably dependent upon the methods used and the individual study, we have decided that a lower and upper range of molecular weights for the insect viruses relative to those for T4 DNA will be given in this study: 108×10^6 to 130×10^6 as the reported molecular weight range for T4 DNA (8). As can be seen from the sedimentation data and Table 1, all of the insect virus DNAs have approximately the same molecular weight and are very likely

TABLE 1. *Sedimentation coefficients and molecular weight estimates relative to T4 DNA[a] sedimentation*

RPM	TnGV		SfGV		TnSNPV		RMNPV		SfMNPV	
	$s_{20,w}$	Mol wt × 10^6	$s_{20,w}$	Mol wt × 10^6	$s_{20,w}$	Mol wt × 10^6	$s_{20,w}$	Mol wt × 10^6	$s_{20,w}$	Mol wt × 10^6
62,500,										
linear	60*S*	99–119	59*S*	95–114	60*S*	99–119	59*S*	95–114	59*S*	95–114
circular	80*S*		76*S*		75*S*		73*S*		75*S*	
50,000,										
linear	59*S*	95–114	58*S*	91–109	58*S*	91–109	58*S*	91–109	59*S*	95–114
circular	71*S*		66*S*		65*S*		66*S*		66*S*	
40,000,										
linear	59*S*	95–114	59*S*	95–114	59*S*	95–114	58*S*	91–109	58*S*	91–109
circular	66*S*		60*S*		61*S*		59*S*		59*S*	

[a] Lower and upper limits of $s_{20,w}$ and molecular weights based upon those reported for T4 when using the size ranges from 108 to 130 × 10^6 (7). Estimates were calculated using $S_1/S_2 = (M_1/M_2)^{0.38}$ The $s_{20,w}$ for T4 = 62. All sedimentation coefficients shown are ±2*S* relative to T4, with all values shown an average of at least three determinations.

indistinguishable by sedimentation behavior in neutral sucrose gradients by such estimates and techniques.

Some of the values reported for 40,000 rpm centrifugation are perhaps not as valid as compared with results obtained at higher speeds of centrifugation, because of the inability to accurately measure the position of the linear form of DNA, but are estimates derived from positions in parallel gradients relative to that of T4 and T7 DNA. Because of this difficulty of providing accurate measurements of relative co-sedimentation rates for the insect virus DNAs, a ratio system using T7 and T4 DNAs was employed to provide additional confidence in the parallel and co-sedimentation studies. At 62,500 rpm, T4 sedimented 1.42 ± 5% the distance relative to T7 DNA. At 40,000 and 50,000 rpm, T4-T7 was 1.86 and 1.75, respectively. Although the variability of the measurements did not warrant the assigning of more absolute estimates of sizes to specific DNAs, the data (Table 1, Fig. 2) clearly show that all linear forms of the insect virus DNAs at speeds of 62,500 rpm or less sediment 2 to 4*S* slower as compared with that of T4 DNA. Please note that the relative rate of DNA sedimentation for T4-T7 increases with a decrease in rotor speed. Sedimentation of linear forms of insect virus DNAs conformed with these ratios relative to T7, but once again consistently at a 2 to 10% lower ratio. Sedimentation of linear insect virus DNAs relative to T4 DNA, however, gave more consistent results for comparison.

Although the sedimentation dependence behavior of the circular DNA was not carefully studied here, the results obtained from using the preparative ultracentrifuge are interesting from the standpoint of the initial characterization of those DNAs. After sedimentation in neutral sucrose gradients constituted in salts providing a high ionic strength, we have not been able to distinguish differences in behavior between closed and relaxed circles. As more carefully shown with other circular species of DNA (4, 23) sedimentation behavior and the dependence upon ionic strength and rotor speed is an extremely complex relationship and difficult to analyze by using an analytical ultracentrifuge. The faster of the sedimenting DNAs in this study is therefore a mixture of the two forms. At the speed of 62,500 rpm, the ratios of sedimenting distances of circular to linear DNA was approximately 1.22 to 1.28 (lower and upper limits observed) for all GV and NPV DNAs; at 50,000 rpm, this ratio was reduced to 1.13 to 1.16. At 40,000 rpm, the ratio was very difficult to determine by co-sedimentation because of the close proximity of the two peaks, but it is in the range from 1.0 to 1.1.

The covalently closed DNA can assume a different tertiary configuration and therefore exhibits significantly different sedimentation properties under conditions of low ionic strength. We have confirmed (shown for SfMNPV and RMNPV in Fig. 3) in our studies that the amount of covalently closed DNA (superhelix in low-salt gradients) to relaxed circular DNA can range from 30 to 60% of that recovered from the freshly prepared covalently closed DNA that can be observed in Fig. 1 as the heavy band. At 62,500 rpm, the superhelical DNAs sedimented at approximately 85 to 95*S* ± 3*S*, relative to the insect virus linear DNA and T4 DNA. The sedimentation behavior of relaxed and covalently closed circles is quite concentration dependent. The most consistent and reliable *S* values were obtained in this

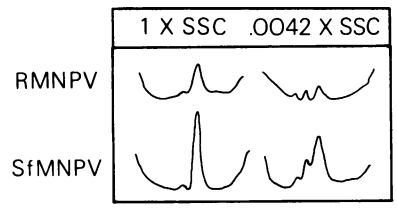

FIG. 3. *Sedimentation in relation to ionic strength conditions in the sucrose gradients. RMNPV and SfMNPV DNA was recovered from the heavy band purified on ethidium bromide-CsCl gradients. Five to 20% sucrose gradients were made up in 1× SSC and 0.0042× SSC, pH 7.0, with I = 1.1 and I = 0.001 (0.0042× SSC), respectively. In the gradients with a salt concentration giving an I = 0.001, all three forms of the DNA, linear, relaxed circles, and covalently closed (superhelix) DNA can be observed in the respective positions. Centrifugation was carried out at 10 C and 62,500 rpm for 70 min. Note that some degradation of the superhelix DNA to relaxed circles and linear forms was unavoidable regardless of care of handling. Approximately 10 μg of DNA was loaded onto each gradient.*

study by using 5 μg or less of DNA in those sedimenting bands.

Alkaline sucrose gradient centrifugation. Alkali denaturation and centrifugation with the GV and NPV DNAs provides additional and very reproducible evidence that the NPV DNAs are slightly smaller than the GV DNAs, and the GV DNAs are slightly smaller than denatured T4 DNA. This is not distinctly obvious from the data derived from neutral sucrose gradient centrifugation. The results of alkali gradient centrifugation and the estimates of molecular weights from such experiments are presently difficult to interpret, because it has been shown that the sedimentation behavior of high-molecular-weight nucleic acids under conditions of high pH is somewhat variable and, perhaps, not predictable (15). Denatured interlocked circular rings of TGV DNA sediment slightly faster than those covalently closed forms of the nuclear polyhedrosis virus DNAs (Fig. 4). As shown, TnSNPV, RMNPV, and SfMNPV DNAs are not distinguishable by this sedimentation behavior, but are distinctly different from the TnGV DNA.

Figures 5 and 6 show the results of alkali sucrose gradient centrifugation after 60 min. These studies were conducted with individual nuclear polyhedrosis virus DNAs as compared with T4 DNA (Fig. 5) and TnGV DNA (Fig. 6). Numerous centrifugations demonstrated that the most reliable sedimentation behavior at 62,500 rpm could be obtained for centrifugation periods between 60 and 80 min (Fig. 7). Denatured T4 DNA sediments slightly faster, approximately 2 to 3%, than the single-strand circular form of the nuclear polyhedrosis virus DNA (Fig. 5). TnSNPV, RMNPV, and SfMNPV once again were very similar and could not be distinguished by sedimentation behavior. Figure 6 shows the results of parallel and co-sedimentation of each NPV DNA and TnGV DNA. The GV DNA single-stranded linear species sediments to the same level as that of the single-strand circles of the nuclear polyhedrosis virus DNAs, thus showing that in alkaline sucrose the granulosis virus DNA is apparently larger than those of the NPVs. The error of reproducibility of our measurements by using the specified speed of centrifugation and times shown was consistently demonstrated to be 2 to 3%. Relative ratios of distances of sedimentation showed that TGV linear DNA sedimented approximately 5 to 8% (ratios of 1.05 to 1.08; T4-GV DNA) slower than dena-

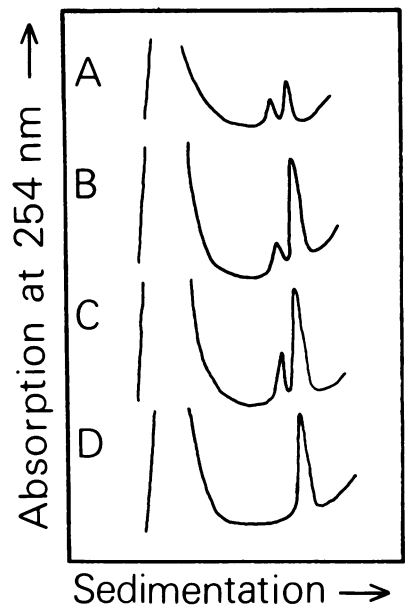

FIG. 4. *Relative and co-sedimentation of covalently closed TnGV DNA and NPV DNAs in alkaline sucrose gradients. The gradients were 2 to 40% sucrose constituted in 1.0 M NaCl plus 0.3 M NaOH plus 10 mM EDTA. Centrifugation was conducted at 62,500 rpm for 20 min (1,200 s from the start until breaking action of the centrifuge) at 20 C. The faster of the sedimenting peaks in each gradient is TnGV DNA. (A) SfMNPV plus TnGV; (B) RMNPV plus TnGV; (C) TnSNPV plus TnGV; and (D) TnGV DNA only. Scanning patterns represent 2 to 3 μg of DNA per gradient. Absorption at the top of the tube represents unresolved denatured single-strand linear and single-strand circular DNA.*

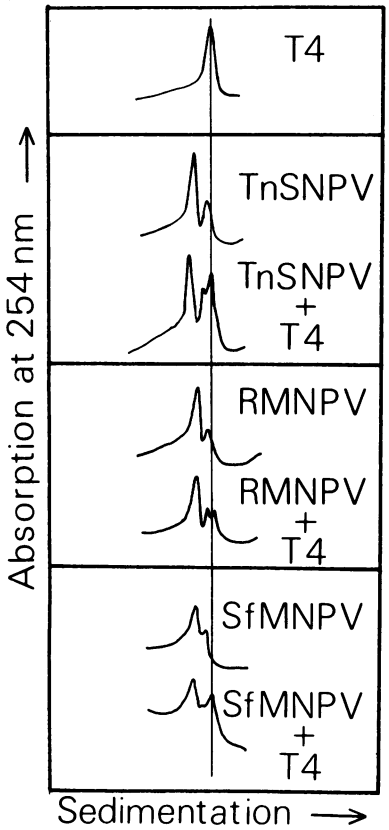

FIG. 5. *Parallel and co-sedimentation of NPV DNAs relative to T4 DNA in alkaline sucrose gradients. Gradient conditions and DNA concentrations were the same as described in Fig. 4. Centrifugation was for 60 min.*

tured T4 DNA. All NPV denatured linear DNA reproducibly sedimented 15 to 18% (ratio of 1.15 to 1.18; T4-NPV DNA) slower than T4 DNA and 7 to 10% slower than GV DNA. Regardless of the species of insect virus DNA, the circular form always sedimented 9 to 10% faster relative to the linear form. The effect of rotor speed on sedimentation dependence of linear and circular insect virus DNAs was determined by centrifugation to the same approximate region of an alkaline gradient at 40,000 and 50,000 rpm for comparison with that of 62,500 rpm. Relative to T4 DNA, only a reduction of a ratio of 1.08 to 1.05 of T4:linear TnGV was observed, and sedimentation dependence of the circular species on rotor speed was not significantly demonstrated, as was shown to occur under conditions of neutral sedimentation.

The region of the central two-thirds of the gradient has been utilized in our alkaline sedimentation studies because this gives well-resolved peaks, and distances of sedimentation relative to time have been empirically determined to be approximately proportional by using TGV DNA, T4, and T7 in that region. Figure 7 shows a series of sedimentations (points averaged) at given times for parallel and co-sedimentation of the linear forms of TnGV

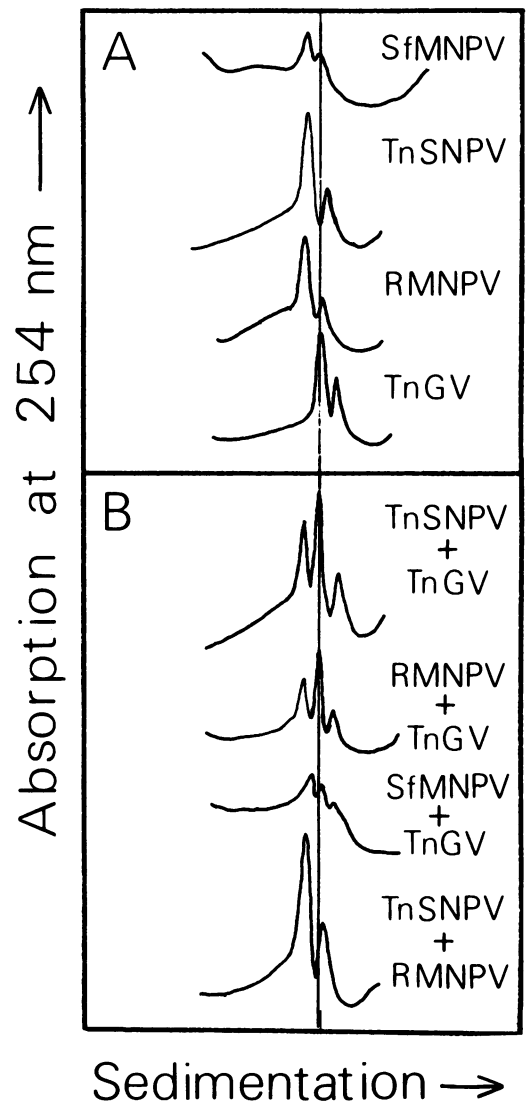

FIG. 6. *Parallel and co-sedimentation of NPV DNAs relative to TnGV DNA in alkaline sucrose gradients; gradients and conditions were similar to those described in Fig. 4 and 5. (A) Observation of the results of parallel sedimentation; and (B) observation of the results of co-sedimentation with TnGV DNA. Note in the last scan, panel B, that TnSNPV and RMNPV are indistinguishable in observed sedimentation behavior.*

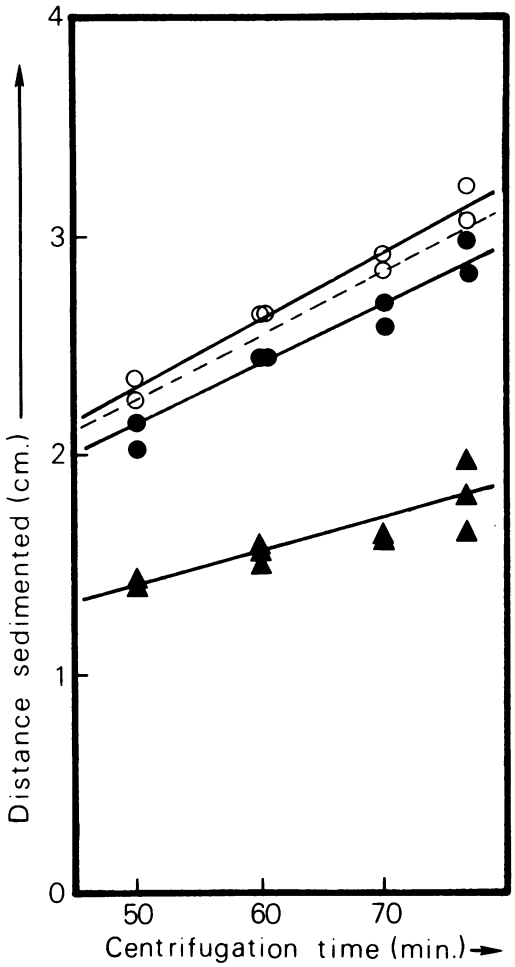

FIG. 7. *Denatured single-strand circular and single-strand linear DNA sedimentation in alkaline sucrose gradients as compared with denatured T4 and T7 DNAs. In this figure: △, T7 DNA; ●, denatured linear TnGV DNA; ○, denatured circular TnGV DNA; -----, relative position of denatured T4 DNA sedimentation. The interlocked rings arising from denatured, covalently closed DNA have pelleted in these centrifugations. The time of centrifugation was chosen from the start of the run until manual shut-off at the designated time on the graph. Each point shown is an average of at least two centrifugations. Centrifugation was conducted at 62,500 rpm and 20 C and the times shown by using an SW65 rotor and L265B preparative ultracentrifuge. Alkaline gradients were prepared as described in Fig. 4 with 2 to 3 μg of DNA consistently used per run.*

and T4 and T7 DNA. The apparent sedimentation rate is proportional to $s_{20,w}$ (sedimentation coefficient corrected to water at 20 C). If the lines are extended, they appear to radiate from a point that is near the top of the gradient and near the start of the centrifuge run. From such plots, the apparent sedimentation coefficients can be obtained in three ways: (i) by determining how far the DNA sedimented in a given time from its apparent starting point; (ii) by determining the time of sedimentation to a given position; and (iii) by using the slope of the plot of distance versus time (rate) that can be normalized to a known value for T4 ($s_{20,w}$ = 72.7*S*). Table 2 shows the results of these calculations and the estimates of molecular weights derived by using the latter technique and the empirical equation of Studier (18) for sedimentation in alkaline solutions. As mentioned previously, the validity of such estimates is questionable on both theoretical and empirical grounds, but nonetheless this study provides a useful system for characterization of the insect virus DNAs and an initial understanding of behavior in alkaline sucrose gradients. Therefore, alkaline gradient sedimentation studies are presented in this study not as evidence of absolute differences in molecular weight as compared with bacteriophage standards, but to provide an additional experimental tool for the empirical characterization of granulosis and nuclear polyhedrosis nucleic acids relative to established marker systems.

Thermal melting profiles. The thermal denaturation profiles for TnSNPV, TnGV, and SfMNPV DNA are compared to previously reported T_m values for GV DNAs (Fig. 8). All measurements were made relative to T7 DNA. RMNPV is not shown but is identical to TnSNPV. The thermal melting points for these nucleic acids are summarized in Table 3. The results show that in terms of base composition, the nuclear polyhedrosis virus DNAs of *T. ni* are similar, but there are differences in G+C content as compared with the granulosis viruses of their respective hosts. Comparative studies using equilibrium banding in CsCl did not show significant differences.

TABLE 2. *Sedimentation coefficients and molecular weight estimates from alkaline sucrose gradient centrifugation*[a]

DNA	$S_{20,w}$	Mol wt_{ss} (10^6)	Mol wt_{ds} (10^6)
T4	73*S*	67	134
TnGV	65*S*	52	104
SfGV[b]	62*S*	48	96
NPVs	59*S*	42	84

[a] Molecular weight estimates were calculated from Studier (18): $S_{20,w} = 0.0528\ M^{0.4}$.

[b] Calculations are from results reported by Summers and Anderson (19, 20).

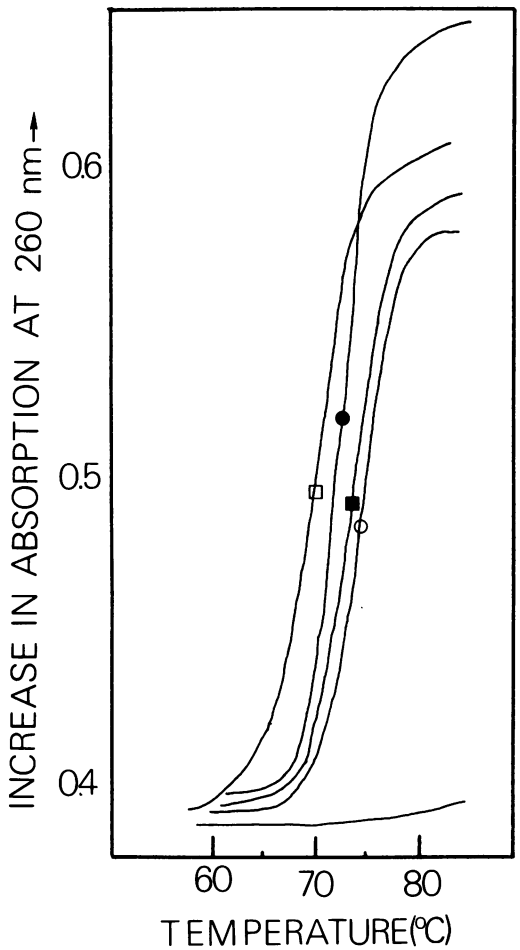

FIG. 8. *Thermal denaturation profiles comparing GV and NPV DNAs. Melting points were determined in 0.1 × SSC (0.015 M NaCl plus 0.0015 M sodium citrate, pH 7.0) in a Gilford model 2400 recording spectrophotometer. T7 DNA was employed as the standard, and T_m values were calculated from the formula of Mandel and Marmur (13) by using the T_m for T7 DNA reported by Szybalski (22; 50% G+C and a calculated T_m of 74.5 C). The DNA was purified as described in the text. These preparations did not contain covalently closed DNA. □, TnGV DNA, T_m of 69.3 C. ●, TnSNPV DNA, T_m of 70.5 C. ■, SfGV DNA, T_m of 73.4 C. ○, SfMNPV DNA, T_m of 74.5 C. RMNPV is not shown but is identical to that profile given for TnSNPV DNA.*

DISCUSSION

The results of this study very closely parallel the previous studies by Summers and Anderson (19, 20) on the hydrodynamic properties of granulosis viruses DNAs. The NPVs studied herein have been shown to be similar in size to the GV DNAs, although perhaps slightly smaller as estimated by sedimentation behavior in alkaline sucrose gradients. All DNAs have been estimated to be slightly smaller than bacteriophage T4 DNA standards, but are not likely to be smaller than 10% in size. The population of molecules as released and purified from the NPVs is very similar to that shown previously for GV DNAs. The DNA, as gently released from the virus and sedimented in sucrose gradients, consists of approximately 20 to 30% covalently closed DNA, approximately 60% relaxed circles, and less than 10% as the linear duplex. Although there are structural and sedimentation similarities in neutral sucrose, the thermal denaturation profiles demonstrate distinct differences in G+C content with the exception of TnSNPV as compared with RMNPV. This latter observation is interesting because these two DNAs are indistinguishable by any studies utilized herein, and yet one is an NPV that has multiple numbers of nucleocapsids within a single envelope (RMNPV), and the other (TnSNPV) consists of singly enveloped nucleocapsids. However, with both, many enveloped nucleocapsids or enveloped bundles of nucleocapsids are occluded in large protein crystals. Although RMNPV was originally reported in an infection of *R. ou* larvae, it is highly infectious for *T. ni* larvae. Differences between the hydrodynamic behavior of GV DNAs and NPV DNAs can be shown in alkaline sucrose gradients, with that sedimentation behavior being extremely reproducible. The GV DNAs sedimented slightly slower than denatured T4 DNA standards, however, all NPVs sedimented 15 to 18% slower than T4 DNA and approximately 10% slower than GV DNA. Studies are in progress using the Kleinschmidt technique to determine the sizes of these molecules to clarify the apparent differences in sedimentation behavior.

In these studies, little evidence was observed of the presence of a significant amount of DNA released directly from the virus sedimenting at approximately 40 to 45*S*. There was always some absorption at the top of the tube in the 12

TABLE 3. *G + C content*

Virus or host	T_m	G + C (%)
Tn Host	68.5	36
TnGV	69.3	37.5
TnSNPV	71.5	43
RMNPV	71.5	43
Sf host	69.5	38
SfGV	74.5	50
SfMNPV	72.8	46

to 14S region. The nature and/or significance of that DNA and whether it may be liberated directly from the virus particles and not from a degradation product is not known at this time. However, it is suspected that the lower-molecular-weight DNA is very likely nothing more than degraded products due to handling and breakage; nevertheless, this will need confirmation. This is important because of recent reports (6, 11, 17, 25) on the infectious properties of other NPVs both in vivo and in vitro. Several investigators have isolated highly infectious DNA from infected silkworm moth tissues for a comparison with infectious DNA isolated from purified virus (6, 11, 25). They demonstrated, by using the phenol-detergent method, that the largest size molecules that could be isolated and detected by hydrodynamic studies from the virus were approximately 37S or 31×10^6. They also observed considerable heterogeneity in their preparations, showing DNA sedimenting at 140, 94, 61, 45, and 14S. Those sedimentation coefficients below 140S correspond nicely to those in this and previous reports by Summers and Anderson (19, 20) showing that in higher ionic strength, gradients relaxed circles and covalently closed DNA sediment at approximately 65 and 90S with the linear duplex at 60S. A discontinuous distribution of molecules ranging from 60 to 40 or 45S was not observed. Also, TnGV DNA, after shearing with a syringe, produced a band of DNA sedimenting at approximately 12 to 14S. In the report of Summers and Anderson, it was desmonstrated that the trailing edge of the 60S DNA upon natural or artificial degradation showed a continuous relationship to degradation and not a discontinuous one. However, under higher concentrations of DNAse I, the DNA nearly all converted in a broadly sedimenting 40 to 45S DNA considered to be an average of degraded half molecules (19, 20). Kok et al. (11) observed, in their DNA preparations purified by phenol-detergent extraction procedures, circular forms ranging in size from 30 to 50 μm in length and giving molecular weight estimates of 58 to 117×10^6. Superhelical forms were not observed, although circular, relaxed forms of DNA were observed in concentrations of 10 to 15%. However, the results obtained from hydrodynamic studies demonstrated the presence of DNA with an upper size limit of 37S. Because they demonstrated in their studies that the DNA molecules are units of, or multiples of 5 μm, the question of subgenomic infectivity has been postulated. Support for this is also provided from other studies, most recently one by Zherebtsova et al. (25), by using the NPV of *Galleria mellonella*. Low-molecular-weight infectious entities have been reported as early as 1943 (10) from NPV-infected *B. mori* and more recently by Aizawa (1) and Barefield and Stairs (2). In addition, there have been many reports of the difficulty involved in infecting in vitro insect cell lines with purified virions. Infectivity is more efficiently produced if infected hemolymph is used, because the nature of the infectious material is not yet characterized (6, 24). Nevertheless, the subject of subviral infectivity is yet to be resolved. It is hoped that the studies provided herein will give additional confidence and standardized methods for the purification, characterization, and study of virus-host cell interactions that will ultimately lead to a characterization and accurate understanding of virus infections in insect cells.

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