

Biochemical, Biophysical, and Biological Properties of Densonucleosis Virus (Parvovirus)

II. Two Types of Infectious Virions

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Among the infectious densonucleosis virus (DNV) particles, two types of virions, DNV-I and DNV-II, have been identified. The density of DNV-I was about 1.40 g/ml and depended on the pH, whereas the density of DNV-II was independent of the pH and was 1.44 g/ml. The sedimentation rates of these particles were 111S and 89S, respectively. The specific extinction coefficients and the absorption spectra, corrected for light scattering, were also different for these two types. The electrophoretic mobility of both types of virions was identical under the experimental conditions used. Both types of particles contained single-stranded DNA with similar physicochemical properties. The difference between these types of virions seemed to be due to a different protein content, DNV-II containing about 600,000 daltons less protein, and to a different quaternary structure as indicated by the change in the density of DNV-II, in the presence of divalent cations, without any change in the density of DNV-I.

Densonucleosis virus (DNV) belongs to the family of the *Parvoviridae* (1, 11, 18, 19), which contain linear, single-stranded DNA (molecular weight, 1.5×10^6 to 2.2×10^6) and have three main proteins of relatively high molecular weight (50,000 to 100,000). One of these proteins is present as 60 copies per virion, the other proteins in significantly lower numbers.

Although most of the parvoviruses contain only one specific strand of the replicative form (5, 17), both the adenovirus-associated virus and DNV contain single complementary strands in separate virions (12, 15). The plus and minus strands of these two viruses readily hybridize in vitro to form a double-stranded structure (1, 2, 11, 12, 17). This property may reflect a different mode of replication for these two viruses and necessitates further work to decide whether they form a separate genus among the *Parvoviridae*. DNV is the only virus known to possess both autonomous replication and separately encapsidated complementary single-stranded DNA (11, 12).

Concerning the buoyant density of DNV virions, conflicting results have been reported, giving values of 1.40 g/ml (19) and 1.44 g/ml (5). Previous observations in this laboratory indicated that virions of both densities exist (18), a phenomenon also observed for some other parvoviruses (5). It has been suggested that this difference in the buoyant densities of parvoviruses might be due to the loss of a protein layer

(5), to a lower content of minor proteins (6), or to a proteolytic conversion from the dense to the lighter type (3).

The purpose of the present study was to characterize these two types of virions with respect to their structure and properties.

MATERIALS AND METHODS

Extraction and purification of the virus. A modification in the extraction procedure previously used (18) was the addition of 1 volume of chloroform-butanol (1:1) or carbon tetrachloride to 2 volumes of buffer, containing a few crystals of 1-phenyl-2-thiourea. The purification method was also modified from the earlier procedure by increasing the pH used for extraction to at least 7.5 (with 50 mM Tris-hydrochloride buffer) since most of the virions have an apparent isoelectric point of 6.5 (as determined with an LKB Ampholine column type 8101; unpublished data). In some experiments the buffer was supplemented with 5 mM NaHSO₃ (proteolytic inhibitor).

The larvae were homogenized in this mixture (1 ml of medium for 2 larvae) with a Waring blender 702 CR for 45 s. The phases were separated by centrifugation at 10,000 rpm for 10 min in an SS-34 rotor in a Sorvall RC2-B centrifuge. The aqueous phase was then centrifuged for 1 h at 16,000 rpm to remove large particles. The virus was pelleted from the supernatant by centrifugation for 1.5 h at 41,000 rpm in an A321 rotor in an IEC-B60 ultracentrifuge. The sediment was resuspended in 0.5 ml of the buffer. Any unsuspended material was removed by centrifugation for 5 min at 10,000 rpm. The superna-

tant was finally filtered through cellulose acetate filters of decreasing pore size (type EA, 1,000 and 200 nm; Millipore Corp.).

Sucrose density gradients and isopycnic centrifugation in CsCl. Preparation of the linear sucrose gradients, conditions of the centrifugation, analysis of the gradients, and collection of the fractions were as described before (18). Virus was centrifuged to equilibrium in CsCl gradients (in the buffer used for extraction) by the method described previously (18).

Determination of the buoyant density and the S value of the virus. The position of the virus bands in the tubes was determined, and the tubes were punctured at 3-mm intervals from top to bottom. The refractive index of each sample was measured with an Erma refractometer, and the density of the virus was established from the regression curve of the medium.

Virus suspensions in 0.1 M NaCl + 0.01 M Tris hydrochloride were analyzed in a Beckman model E analytical ultracentrifuge at 30,000 rpm. Ultraviolet scans were taken at 4-min intervals.

Influence of ionic strength and pH on the purification of the different DNV types. In a series of experiments, the ionic strength of the buffer used for extraction was increased by the addition of 0.06 or 0.12 M NaCl, and the pH was adjusted to 7.5, 8.0, 8.5, or 9.0.

Effect of divalent cations on DNV types. Purified DNV types were dialyzed against 0.05 M Tris-hydrochloride + 0.06 M NaCl, pH 8.0, containing either 10 mM EDTA or 20 mM Mg^{2+} + 5 mM Ca^{2+} , and were subsequently analyzed on CsCl gradients.

Specific extinction coefficient and absorption spectra. A known quantity (in units of absorbance at 260 nm [A_{260}]) of the purified virus was dried to constant weight after extensive dialysis against distilled water. The weight of the sample was established to 10^{-5} g with a Mettler Gram-atic balance. The specific extinction coefficient is expressed in terms of A_{260} and ($A_{260} - A_{290}$) units, since absorbance of the viral proteins roughly equals its A_{260} value in the range of 287 to 290 nm.

A concentrated suspension of highly purified virus (about 100 A_{260} units per ml) was diluted before use with 50 mM Tris-hydrochloride buffer, pH 7.5, and filtered through cellulose acetate filters of 1,000- and 200-nm pore size (Millipore Corp.). The absorption spectra were obtained with a Beckman DU spectrophotometer and were corrected for light scattering. The scattering being proportional to $1/\lambda^n$ where $n < 4$ (16), the value of n was determined from light scattering in the range of 315 to 600 nm.

Determination of the protein content of the DNV types. The virus was diluted with 0.1 N NaOH, and the quantity of protein per A_{260} unit was determined by the method of Lowry et al. (13), using twice-recrystallized ovalbumin as reference protein.

Polyacrylamide gel electrophoresis. Large-pore gels were prepared by polymerizing 4% polyacrylamide containing 15% bisacrylamide in 0.375 M Tris-hydrochloride buffer, pH 8.3, using 0.03% N,N,N',N' -tetramethylethylenediamine and 0.07% ammonium persulfate as catalysts.

The virus preparation was suspended in Tris-gly-

cine buffer (0.04 M with respect to glycine), pH 8.0, with 5% sucrose, and about 20 μ g was applied to each gel. Electrophoresis was performed in the Tris-glycine buffer (without sucrose) for 4 h at 6 mA per gel in a Buchler polyanalyst at room temperature.

The gels were stained with a 0.25% solution of Coomassie blue 250 G in 9% acetic acid and 45% methanol for 1 h and destained electrophoretically in a solution of 7.5% acetic acid and 5% methanol.

Sucrose gradient analysis of DNA of DNV. The DNA of the virus was released by sedimenting the purified virus into a 5 to 20% sucrose gradient containing 0.3 N NaOH, 0.15% Sarkosyl, 0.7 M NaCl, and 1 mM EDTA, as described elsewhere (10). The gradients were centrifuged in an SB-405 swinging-bucket rotor for 3 h at 30,000 rpm at 4°C and analyzed as described previously (18).

Staining with acridine orange. The virus preparation was dried on slides, fixed in Carnoy solution, and stained as described elsewhere (14).

Reaction with diluted formaldehyde. Diluted formaldehyde was added to purified virus preparations to a final concentration of 1.8%, and the hyperchromicity was measured after 1, 6, and 24 h.

RESULTS

Purification of infectious DNV types. On centrifugation in a sucrose gradient, the DNV preparation gave rise to two poorly separated components (Fig. 1). The fast-sedimenting virus has been designated as DNV-I and the slower-moving particle as DNV-II. The resolution between these two components is poor, and consequently the type II component, which is present in a lower concentration, is heavily contaminated with type I. Subsequent isopycnic centrifugation of these components in CsCl yielded a buoyant density of about 1.40 g/ml for DNV-I and of about 1.44 g/ml for DNV-II (Fig. 2). A similar profile was obtained when the sucrose gradient step was omitted and the virus suspension was centrifuged directly to equilibrium in a CsCl gradient. The resolution between the two bands in CsCl was very good, and since the bands were clearly visible, they could be recovered by puncturing the tube at the appropriate place.

The sedimentation rates, determined in 0.1 M NaCl + 0.01 M Tris-hydrochloride, pH 7.5 or pH 8.0, at 20°C were 111S for DNV-I and 89S for DNV-II.

Both types of particles were highly infectious, exhibiting high infectivity up to a dilution of at least 10^{-10} . However, it is difficult to establish accurately a comparable 50% lethal dose for these two types since it is rather tedious to determine the exact instar of the larvae. Moreover, at high dilutions of the virus the progression of the disease is slow, and so pupae appear. In addition, some of uninfected larvae do not form butterflies, a finding that renders it

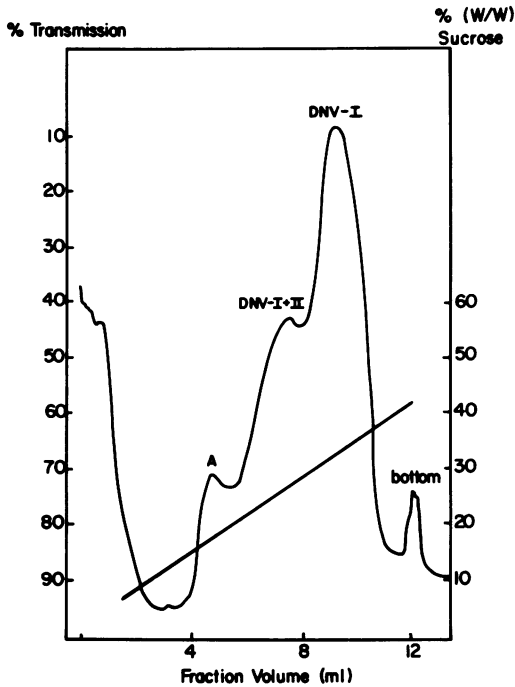


FIG. 1. Velocity gradient centrifugation of DNV in sucrose. Conditions and analysis were as mentioned in Materials and Methods. The A band contains mainly empty particles.

difficult to estimate quantitatively the fraction that died of the viral infection.

Modifications in the extraction and purification of DNV. The extraction procedure was modified with respect to the pH of the medium and the organic solvent used. The results of these modifications are given in Table 1. To minimize the effect of the absorbance of contaminating proteins, the yield (after CsCl centrifugation) was expressed in ($A_{260} - A_{290}$) units (Table 1). The slightly higher pH of the buffer used for extraction increased the yield significantly over the conventional method. Similarly, carbon tetrachloride was also found to improve slightly the yield over chloroform-butanol; however, the purity of the preparations decreased.

Influence of the ionic strength and pH on the purification and density of the DNV types. Buffers at three different ionic strengths and four different pH values were assayed. The virus was banded on CsCl gradients and the bands were extracted as described in Materials and Methods. The quantities of virus recovered were expressed in A_{260} units (after dialysis).

The pH had a clear effect on the total virus yield and the percentage of each type (Fig. 3). At a pH higher than 7.5, the virus yield was

lower, particularly at $\text{pH} \geq 8.5$. However, the fraction of DNV-II increased with increasing pH and, in absolute terms, the quantity of DNV-II reached a maximum at about pH 8.0 and then decreased gradually. The addition of different quantities of NaCl to the buffer

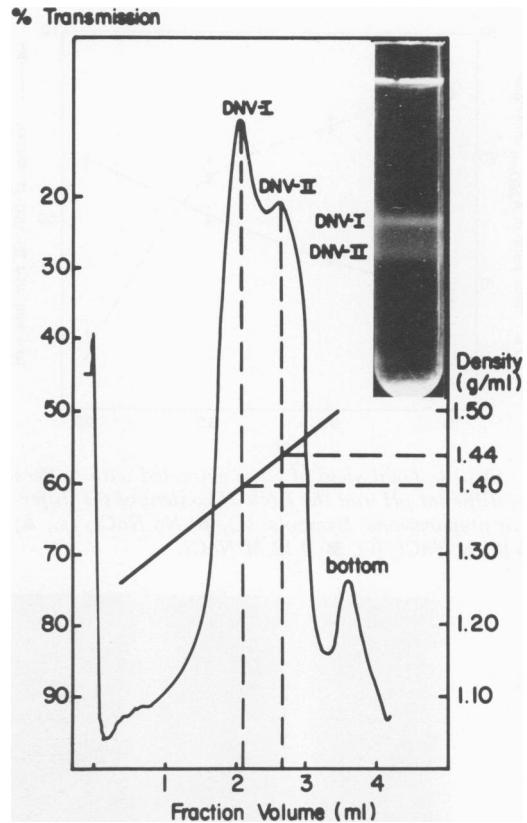


FIG. 2. Isopycnic centrifugation of the type I and type II particles recovered from the sucrose gradient. The bottom of the tube was punctured, and the CsCl gradient was pushed upwards through an IEC flow cell attached to the spectrophotometer. Better separation of the bands was obtained by puncturing the sidewall of the tube and withdrawing the clearly visible bands with a syringe.

TABLE 1. Yield and absorbance ratio (A_{260}/A_{240}) of the virus extracted by the conventional or the modified method

Method	Yield in ($A_{260} - A_{290}$) units/larva		A_{260}/A_{240}	
	DNV-I	DNV-II	DNV-I	DNV-II
Conventional	0.02	0.03	1.50	1.62
pH 7.5; chloroform-butanol	0.13	0.21	1.52	1.66
pH 7.5; carbon tetrachloride	0.17	0.25	1.40	1.57

showed no significant effect. The density of DNV-I at the apparent isoelectric point (pH 6.5) was 1.395 g/ml and increased at higher pH (pH 7.5, 1.400 g/ml; pH 8.0, 1.409 g/ml; pH 8.5, 1.420 g/ml). On the other hand, the pH did not show a marked influence on the density of DNV-II. Changes in the ionic strength used in the ex-

traction procedure had an influence only on the density of DNV-II. With the addition of 0.06 or 0.12 M NaCl, the density of DNV-II increased from 1.440 to 1.446 and 1.453 g/ml, respectively.

Influence of divalent cations and EDTA on the density of DNV. The addition of 20 mM $MgCl_2$ + 5 mM $CaCl_2$ to the purified DNV types in Tris-hydrochloride buffer had a profound effect on DNV-II but not on DNV-I. The density of DNV-II decreased to 1.415 g/ml (Fig. 4[6]), whereas its sedimentation rate increased to 106S. With these changes, DNV-II became almost indistinguishable from DNV-I (Fig. 4[1]) and was, therefore, designated as DNV-I' (Fig. 4[6]). This effect was found to be reversible with 10 mM EDTA.

It was not possible to obtain a similar transformation from DNV-I. However, in the presence of EDTA, DNV-I became less stable, and after 24 h 25% of the DNV-I was broken down, the remaining part giving a more diffused band (Fig. 4[2]).

Specific extinction coefficients, absorption spectra, and protein content. The extinction coefficients for the DNV-I and DNV-II types, at a concentration of 1 mg/ml with an optical pathway of 1 cm at 260 nm, were 6.5 and 7.1, respectively. Expressed in $(A_{260} - A_{290})$ units,

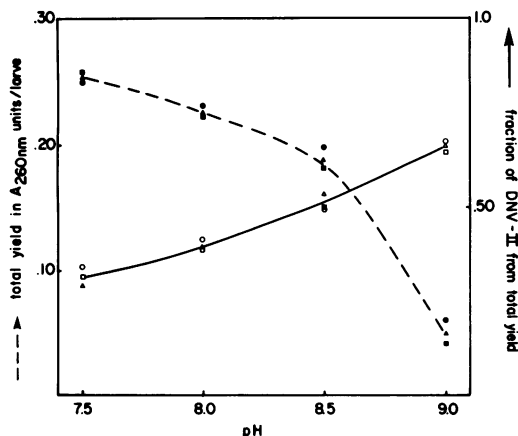


FIG. 3. Total yield of virus extracted with buffers of different pH and the DNV-II content of the different preparations. Symbols: (○, ●) No NaCl; (△, ▲) 0.06 M NaCl; (□, ■) 0.12 M NaCl.

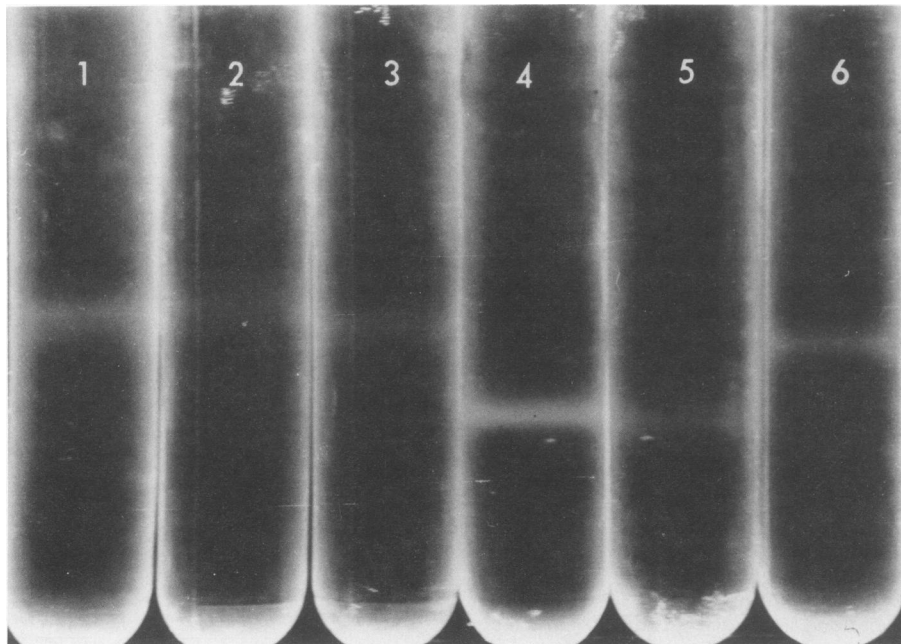


FIG. 4. Influence of divalent ions or EDTA. (1) Type I in standard conditions; (2) type I with addition of EDTA; (3) type I with addition of divalent ions; (4) type II in standard conditions; (5) type II with addition of EDTA; (6) type II after addition of divalent ions. It should be noted that in the presence of divalent cations, type II became almost indistinguishable from type I (tubes 1-3) and was, therefore, designated as DNV-I' (tube 6). Average buoyant density in the tubes was 1.40 g/ml.

these coefficients were 4.6 and 5.0 for types I and II, respectively.

The spectra of both DNV-I and DNV-II at a concentration of 0.1 mg/ml, corrected for light scattering, are given in Fig. 5. Four determinations of the protein content for both types indicated that DNV-I had 103 μg and DNV-II 89 μg of protein per A_{260} unit, or 142.4 and 124.0 μg of protein per $(A_{260} - A_{290})$ unit, respectively.

Polyacrylamide gel electrophoresis. Both DNV-I and DNV-II gave a single band under the experimental conditions described in Materials and Methods (Fig. 6). Co-electrophoresis of the two preparations also produced a single band.

DNV DNA analysis. Sedimentation of the DNA of both DNV-I and DNV-II in alkaline sucrose gradients showed the same characteristics. Both had similar sedimentation properties, and co-sedimentation of the two DNA preparations produced a single peak.

Acridine orange staining showed that both types contained single-stranded DNA (red fluorescence).

Both DNV-I and DNV-II showed hyperchromicity after reaction with diluted formaldehyde, also indicating their single-stranded nature. The absorption maximum shifted from 260 to 263 nm, and the hyperchromicity was 12, 17, and 20% after 1, 6, and 24 h, respectively.

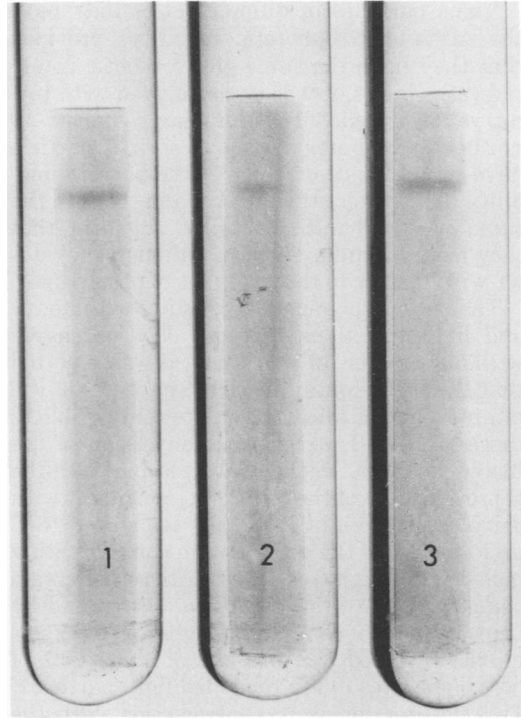


FIG. 6. Polyacrylamide gel electrophoresis of complete virions. (1) DNV-I; (2) DNV-I and DNV-II; (3) DNV-II.

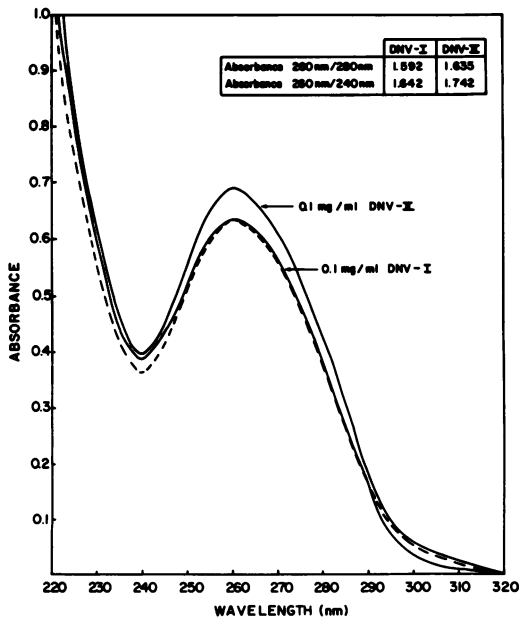


FIG. 5. Absorption spectra of DNV-I and DNV-II corrected for light scattering. The dashed curve represents the spectrum of DNV-II at a lower concentration to correspond to the A_{260} value of DNV-I at a concentration of 0.1 mg/ml.

DISCUSSION

The results of this study could be interpreted as suggesting that the two types of infectious particles differ (i) in their protein content (approximately 600,000 daltons per virion) and (ii) in the balance of forces that determine their quaternary structure.

The first interpretation seems to be supported by the difference in the protein content and in the absorption spectra of the two types of particles, as well as by the higher extinction coefficient for type II than for type I. It is worth mentioning that the difference in the absorption spectra was most significant in the region where mainly proteins absorb (<235 nm). Moreover, the difference in the extinction coefficients would suggest that although the DNA molecules of the two particles are similar, the ratio of DNA protein is different in the two types.

In another study (manuscript in preparation), it was demonstrated that the concentration of the minor proteins p59 and p69 in type II and type I' particles was about one-half of their concentration in type I. A similar phenomenon has also been observed for adenovirus-associated virus type 3 (6). The significance of these findings still remains obscure.

Virus particles of different sizes may have the same electrophoretic mobility, provided that they have the same electrokinetic potential (21). In fact, it was demonstrated with turnip yellow mosaic virus that charges buried 2.0 nm below the outer surface of a virus particle have no influence on their electrophoretic mobility (9). The fact that DNV-I and -II have the same electrophoretic mobility suggests that they have a similar surface, although they differ with respect to their content of p59 and p69.

The only other report (3) dealing with heavy and light infectious particles does not agree with our results. In this study, it was reported that the DNA-containing light particles of the minute virus of mice, which are comparable to those of DNV-I, arise by a conversion of the heavy particles. In the converted particles the B protein (86% of the total protein content with a molecular weight of 72,000) is cleaved to C protein (61% of the total protein content with a molecular weight of 69,000), the conversion being complete in 48 h. It was also stated by Clinton and Hayashi (3) that subsequent experiments revealed the presence of about 20% of heavy particles in the rebanded light particles. This observation is in contradiction with the conclusion that light particles arise from heavy particles by proteolytic action. It is important to note, however, that in the study of Clinton and Hayashi the fate of the particles was followed only during the first 48 h, whereas in the present study virus was harvested from dead larvae 7 to 10 days after inoculation.

The second difference, i.e., the molecular organization and the stabilizing forces in these virions, also seems to play a predominant role in the properties of the two particles. Very little or almost nothing is known about the stability and geometrical organization of the smallest DNA viruses, the parvoviruses. The concept of stability of the quaternary structure is based on the assumption of a minimum free-energy conformation for the viral constituents. The intra-protein interactions maintain a balance of forces required for a state of minimum free energy.

It is evident from the different ways in which pH and ionic strength affect type I and II particles that this balance is different for the two types. It is more difficult, however, to describe accurately the differences in the stabilizing interactions since they comprise all the known short- and long-range noncovalent types of interactions thus far recognized in biological polyelectrolytes. The interactions between the different macromolecules in the DNV virion have been shown to be due to secondary forces only (18). The following observations support

the hypothesis that in type II particles the DNA-protein interactions are relatively more important than in type I: (i) the type II virions are denser than would be expected on the basis of a loss of peptides of the order of 600,000 daltons, suggesting that the DNA-shell protein interactions become more pronounced; (ii) protein-RNA interactions in simple RNA viruses are pH dependent and their breakage around pH 7.0 leads to the expansion of the virion (8); (iii) conversion of the dense to the lighter type of particle by divalent cations might involve neutralization of the DNA's negative charge, thus breaking its interactions with certain proteins; and (iv) empty capsids contain all known structural proteins in the same concentration as in type I (unpublished data). A similar observation was also made for adenovirus-associated virus type 3 (7), whereas for other DNA viruses, such as herpesvirus (4), some core proteins are missing in the empty capsid. These findings make it probable that protein-protein interactions provide the greatest contribution to the stability of type I particles. However, other explanations, such as a possible conformational change of the DNA or a change in the quantity of inhibition water (a better penetration of CsCl in the virion), are also possible.

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