Densonucleosis Virus Structural Proteins

D. C. KELLY,^{1*} N. F. MOORE,¹ C. R. SPILLING,¹ A. H. BARWISE,² AND I. O. WALKER²

Natural Environment Research Council, Institute of Virology, Oxford, OX1 3UB,¹ and Department of Biochemistry, University of Oxford, Oxford, OX1 3QU,² United Kingdom

The protein coats of two densonucleosis viruses (types 1 and 2) were examined by a variety of biophysical, biochemical, and serological techniques. The viruses were 24 nm in diameter, contained at least four polypeptides, were remarkably stable to extremes of pH and denaturing agents, and were serologically closely related. The two viruses could, however, be distinguished serologically and by differences in migration of their structural polypeptides. For each virus the "top component" (i.e., the protein coat minus DNA, found occurring naturally in infections) appeared to have a composition identical to that of the coat of the virus and was a more stable structure. Electrometric titration curves of the virus particles and top components demonstrated that the DNA phosphate in densonucleosis virus particles was neutralized by cations other than basic amino acid side chains of the protein coat. Circular dichroism studies showed that there was a conformational difference between the protein coats of top components and virus particles.

Densonucleosis viruses (DNVs) are small icosahedral viruses, isolated from invertebrates, which contain linear single-stranded DNA, and they share many structural features in common with both the defective and the nondefective parvoviruses isolated from vertebrates (10, 17). Structurally, the DNA contained by DNVs is comparable to that of the defective adeno-associated virus genome and is organized as complementary, separately packaged, linear single-stranded DNA which possesses inverted terminal sequences and a limited nonrandom circular terminal permutation (1, 9, 13, 14). The genome is unlike the nondefective parvovirus genome, which (as typified by minute virus of mice and Kilham rat virus) is organized as a linear DNA molecule (of one polarity) with short palindromic sequences at the ends (2).

Little is known about the structure of the protein coat of DNVs. Tijssen et al. (33) have reported that a DNV of the moth *Galleria mellonella* contains four structural polypeptides with molecular weights of 98,000, 69,000, 58,500, and 49,000 and that the 98,000-dalton polypeptide is a dimer of the 49,000-dalton polypeptide. We have previously shown that the protein coat of DNVs has a molecular weight of 3.55×10^6 and that the molecular weight of the virus particle (5.7×10^6) represents the approximate sum of the protein coat, the genome (molecular weight, 1.95×10^6), and the polyamines (molecular weight, 80,000) which constitute the complete virus particle (13-15). In this study we have examined the protein coats of two DNVs

by examining either the top components (i.e., the virus particles minus DNA and polyamines) or the complete virus particles. We have used a variety of physical methods to examine the polypeptide composition of the coat, the secondary structure of the protein, and the interactions of the coat with the genome. In addition, we have compared the two viruses serologically. The two viruses studied were DNV type 1 (DNV1), isolated from *G. mellonella* (24), and DNV2, isolated from the butterfly *Junonia coenia* (29).

MATERIALS AND METHODS

Virus production and purification. DNV1 was originally isolated by G. Meynadier. DNV1 and DNV2 were provided by C. F. Rivers. DNV1 and DNV2 virus particles and corresponding top components were prepared as previously described (13, 20).

Determination of extinction coefficients and concentrations. DNA was estimated by the diphenylamine method as modified by Burton (3). Calibration curves were prepared with standard solutions of 2-deoxy-D-ribose. RNA was estimated by the orcinol method as modified by Mejbaum (23). Calibration curves were prepared with standard solutions of D-ribose.

Due to lack of material, it was not possible to determine the extinction coefficients of virus particles and top components by the dry weight method. The extinction coefficients were therefore measured indirectly, using a refractive index method to determine protein concentration and estimating phosphorus of virus particles after dialysis into 0.1 M NaCl by the method of Jones et al. (12).

The estimation of protein involved measuring the area of a synthetic boundary of a sample of top com-

ponent photographed with Schlieren optics in a Spinco model E analytical ultracentrifuge and comparing this with the area obtained by the same method at the same bar angle for a standard solution of lysozyme, assuming the refractive index increment of both proteins to be 0.193/g per ml at 435.8 nm (5). The method gave a measure of the protein concentration of the solution of top component. Since the refractive index increment of DNA at 436.0 nm is also 0.193 (27), a similar method was used with solutions of virus particles and top component sedimented appreciably at the speeds used (usually 12,590 rpm), photos were taken at various time intervals, and the measured areas were extrapolated to zero time to obtain the initial area.

Routine analysis for protein was by the Folin method (21) as modified by Eggstein and Kreutz (6). Bovine serum albumin was used as a standard, and a correction was applied by using the previous refractive index method and the Folin method on the same sample. Extinction coefficients of virus particles and top components were obtained by measuring the absorbance of a sample of known concentration in a 1-cm-path-length cell at 260 and 277 nm, respectively. Results were expressed in optical density units per milligram per milliliter for a 1-cm path length. UV spectra of virus particles and top components were obtained at 25°C on either a Cary 14 recording spectrophotometer or a Unicam S.P. 500 spectrophotometer. The extinction coefficients for virus particles and top components were $9.49 \pm 0.32/\text{mg per ml}$ at 260 nm and $1.39 \pm 0.11/\text{mg per ml}$ at 277 nm, respectively.

Electron microscopy. DNV particles and top components negatively stained with 2% (wt/vol) uranyl acetate were examined on an AEI 6B electron microscope as previously described (20).

Analytical ultracentrifugation. Analytical ultracentrifugation was performed as previously described (13), using a Beckman Spinco analytical ultracentrifuge equipped with both Schlieren and absorption optical systems.

Preparation of antisera. Antisera were prepared in both rabbits and guinea pigs. Preimmune sera were obtained from rabbits by bleeding from the marginal ear vein and from guinea pigs by cardiac puncture.

Rabbits were immunized by injecting at weekly intervals 1 ml of virus (containing 1 mg of virus) emulsified with 1 ml of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.) subcutaneously, followed by a similar subcutaneous injection with incomplete adjuvant and finally an intravenous injection of 1 ml of virus. Immune sera were taken 4 weeks later.

Guinea pigs were immunized by two intraperitoneal injections (7 days apart) of 1 ml of virus (2 mg of virus) emulsified with 1 ml of Freund complete adjuvant and were bled by cardiac puncture 3 weeks later to provide immune sera.

Gel immunodiffusion. The reactions between antibody and antigen were examined by performing microtests on glass slides (3 by 1 inch [ca. 7.6 by 2.5 cm]) covered in 0.75% (wt/vol) Ionagar 2 (Oxoid Ltd., London, United Kingdom) in physiological saline containing 0.5% (wt/vol) sodium azide and 0.001% (wt/vol) trypan blue.

Polyacrylamide gel electrophoresis. Three polyacrylamide gel electrophoresis systems were used during these studies.

(i) Polypeptides were analyzed on 16-cm-long 12.5% polyacrylamide slab gels, using the buffer system of Laemmli (19) as previously described (7). Samples were denatured by boiling in 0.1 M Tris-hydrochloride (pH 6.8)–2% (wt/vol) sodium dodecyl sulfate (SDS)–2% (vol/vol) 2-mercaptoethanol–10% (vol/vol) glycerol–0.01% (wt/vol) bromophenol blue. β -D-Galactosidase (molecular weight 132,000), phosphorylase (molecular weight, 97,200), transferrin (molecular weight, 90,000), bovine serum albumin (molecular weight, 69,000), and ovalbumin (molecular weight, 46,000) were used to calibrate the gels for molecular weight determinations.

(ii) Polypeptides were analyzed on 10 and 7% SDS-polyacrylamide cylindrical gels (6 by 100 mm), using a continuous phosphate-buffered system as previously described (16). The same five standards were used for molecular weight determinations.

(iii) Native virus particles and top components were analyzed on 3% polyacrylamide gels (5 by 75 mm) made up in 0.1 M Tris-hydrochloride, pH 9.0, in the absence of SDS. Reservoir buffers contained 0.025 M Tris-glycine, pH 9.0. Samples in reservoir buffer plus 10% (wt/vol) sucrose were applied directly to gels. Electrophoresis was at 5 mA per gel for about 70 min.

Nonreducing gels were run by omitting mercaptoethanol from the system as previously described (25). Sugar residues were detected on proteins by Schiff staining as described by Fairbanks et al. (8).

Proteins were located by Coomassie blue staining as previously described. ^{125}I -labeled protein was located by autoradiography, using Kodirex X-ray film. Coomassie blue-stained gels and autoradiographs were scanned in a Joyce-Loebl microdensitometer equipped with a thin-layer attachment.

Iodination of polypeptides. Iodination of polypeptides was carried out essentially as described by Moore et al. (26). Intact virus or top component was iodinated chemically with chloramine-T or enzymatically with lactoperoxidase. Virus components previously denatured by boiling in 1% (wt/vol) SDS for 2 min were also iodinated by the chloramine-T method.

Iodination catalyzed by lactoperoxidase was performed by adding 10 μl (100 μCi) of carrier-free ^{125}I (Radiochemical Centre, Amersham, United Kingdom), 5 μl of lactoperoxidase (1 mg/ml) (Sigma Chemical Co. Ltd., London, United Kingdom), and 10 μl of freshly diluted 0.05% (vol/vol) H_2O_2 to 100 μl of physiological saline containing 20 μg of virus or top component. The incubation was carried out at room temperature for 30 min and stopped by diluting the reaction 40-fold with physiological saline.

Iodination by chloramine-T was performed by adding 10 μl (100 μCi) of ^{125}I to 100 μl of intact or SDS-disrupted virus (20 μg of protein), followed by 10 μl of chloramine-T (14 mg/ml). The reaction mixture was vigorously shaken for 30 s, and the reaction was terminated by the addition of 20 μl of sodium metabisulfite (28 mg/ml).

All iodinated samples were dialyzed against several 1-liter changes of physiological saline before electrophoresis. Intact virus components were rebanded on

20 to 50% (wt/vol) sucrose gradients made up in 7 mM sodium phosphate (pH 7.2)–0.1 M NaCl. The gradients were run at 35,000 rpm for 16 h in a swing-out rotor (6 by 16.5 ml; M.S.E. Ltd., Crawley, Surrey, United Kingdom).

Circular dichroism spectrometry. Circular dichroism spectra of DNV particles and top components were recorded at 20°C in a Rousell Jouan Dicrographe III, using cells of 0.01-, 0.1-, or 1.0-cm path length. Circular dichroism results are reported in terms of θ , the mean residue ellipticity in units of degrees per square centimeter per decimole. The mean residue weight of an amino acid residue was 108 as determined from amino acid analysis (20).

Continuous electrometric titrations. Continuous electrometric titrations were carried out in a Radiometer microtitration assembly, consisting of a thermostatically controlled glass titration cell and magnetic stirrer. HCl (0.1 N) or 0.1 N NaOH was added from an Agla glass micrometer syringe. pH was measured with an E.I.L. direct-reading pH meter with a glass electrode and a calomel electrode joined by a KCl-salt bridge. Before titration a stream of nitrogen was bubbled through the sample until the pH became constant, to remove dissolved carbon dioxide. During the titration a stream of nitrogen was blown over the sample. All titrations were carried out at 25°C. Titrations were performed on top component (2.5 mg/ml) in 0.1 M KCl and on virus particles (3.75 mg/ml) in 0.1 M KCl below pH 5.7 and in 0.001 M KCl above pH 5.7.

The titration curve of top components or virus particles was constructed by subtracting the solvent titration curve (measured separately and corrected for dilution) from the sample titration. The results are expressed as moles of H^+ dissociated or associated per 100 mol of amino acid residues. To determine if titrations were reversible, the samples were immediately retitrated from high or low pH back to about pH 6.0 with 0.1 N HCl or 0.1 N NaOH.

RESULTS

Electron microscopy. Preparations of pure virus particles and top components of DNV stained with 2% uranyl acetate showed that all preparations comprised small, spherical, presumably icosahedral, particles about 24 nm in diameter (Fig. 1). Some of the virus particles were penetrated by a variety of stain, including uranyl acetate, ammonium molybdate, and sodium tungstosilicate. The extent of stain penetration of top components varied from experiment to experiment, but an appreciable number of particles remained inviolate. Structure was observed, but not in sufficiently fine detail to enumerate capsomeres with confidence. Such structure is shown in Fig. 1.

Serology. Relationships between DNV1 and DNV2 were examined by using antisera prepared in rabbits and guinea pigs against DNV1 and in guinea pigs against DNV2. Microimmunodiffusion tests were performed to conserve

antigens and antisera. Suspensions of both viruses were placed in adjacent wells, and their reactions with the three antisera were monitored. After 48 h adjacent bands of precipitation in the gels became contiguous. When the suspensions were tested against antiserum prepared in rabbits, a tiny spur was formed where the two bands of precipitation against DNV1 and DNV2 met (Fig. 2A). This spur was a continuation of the precipitation band formed by DNV1. To show that this was not an artifact, the same virus suspension was placed in adjacent wells; there were no spurs on the precipitation bands produced (Fig. 2B and C). When the antiserum prepared in guinea pigs against DNV2 was used to compare both viruses, it was found that single bands of precipitation became contiguous, with the band formed by DNV2 continuing into a large spur (Fig. 2D). The guinea pig antiserum prepared against DNV1 was of low titer, but it still gave a visible spur on the band created by DNV1 (Fig. 2E).

The virus particles of DNV1 and DNV2 were serologically identical to their respective top components (data not shown).

Polyacrylamide gel electrophoresis of DNV structural polypeptides. Polyacrylamide gel electrophoresis revealed that the virus particles contained four main structural polypeptides, designated in decreasing molecular weight as VP1 through VP4, as shown in Fig. 3. Adeno-associated virus type 2 polypeptides are shown for comparison. The molecular weights of these polypeptides are indicated in Table 1. Estimates of molecular weight varied with the buffer system and gel concentrations used. The polypeptide compositions of the virus particles and top components were identical for each virus (Fig. 4), though the relative amounts of each polypeptide varied slightly (Table 2 and Fig. 4). Additional polypeptides, intermediate in molecular weight between VP1 and VP2, were sometimes detected by Coomassie blue staining though they were more readily detected by iodination procedures.

Omission of a reducing agent from the gels failed to alter the polypeptide profiles of the components, showing that disulfide bonds are not involved in interpolypeptide interaction. Schiff reagent failed to positively stain any of the polypeptides; this is tentative evidence that the polypeptides are not glycoproteins.

Iodination of DNV polypeptides. Iodination of DNV particles and top components was undertaken to explore the location of the various structural polypeptides within these virus particles. The data presented here are for DNV1 components, though similar results were obtained with DNV2 components. All four main

FIG. 1. DNV particles negatively stained with 2% uranyl acetate, showing generally featureless structure. (a) DNV1 particles; (b) DNV2 particles; (c) DNV1 top components; (d) DNV2 top components; (e) DNV2 particles greatly enlarged to show tentative substructure. Note stain penetration in some, but not all, top components. Bar = 100 nm.

FIG. 2. Serological relationships between virus particles of DNV1 and DNV2 tested by microimmunodiffusion. Wells: 1, rabbit antiserum against DNV1; 2, guinea pig antiserum against DNV2; 3, guinea pig antiserum against DNV1; g, DNV1 antigen; j, DNV2 antigen. Note the small but well-defined spur in A.

FIG. 3. SDS-polyacrylamide gel electrophoresis of the structural polypeptides contained by DNV1 (A), DNV2 (B), and adeno-associated virus (C). The proteins were resolved on a 12.5% slab gel.

structural polypeptides and the minor, fifth polypeptide were efficiently radiolabeled with ^{125}I when chemically iodinated with chloramine-T after disruption with SDS (Fig. 5), and the profile was similar to the Coomassie blue stain profiles. Both lactoperoxidase and chloramine-T radiolabeled the two high-molecular-weight

TABLE 1. Molecular weights of structural polypeptides contained by DNV1, DNV2, and adeno-associated virus type 2 (AAV2)^a

Virus	Mol wt ($\pm$ standard deviation) of:	
	Individual polypeptide	Total polypeptides
DNV1	107,300 $\pm$ 300	281,900
	70,900 $\pm$ 400	
	61,100 $\pm$ 100	
	42,600 $\pm$ 300	
DNV2	109,600 $\pm$ 200	281,000
	70,800 $\pm$ 300	
	58,700 $\pm$ 300	
	41,900 $\pm$ 400	
AAV2	107,300 $\pm$ 200	263,300
	89,100 $\pm$ 200	
	66,900 $\pm$ 100	

^a The peptides were resolved on 12.5% polyacrylamide gels. β -D-Galactosidase (molecular weight, 132,000), phosphorylase (molecular weight, 97,400), transferrin (molecular weight, 90,000), bovine serum albumin (molecular weight, 69,000), and ovalbumin (molecular weight, 46,000) were used as standards.

polypeptides (VP1 and VP2) predominantly and a minor polypeptide intermediate in molecular weight between VP1 and VP2 of both virus particles and top components. This demon-

FIG. 4. Densitometric traces of the polypeptides contained by DNV particles and top components stained with Coomassie blue after electrophoresis on a 10% SDS-polyacrylamide slab gel. (A) DNV1 virus particles; (B) DNV1 top components; (C) DNV2 virus particles; (D) DNV2 top components.

strates that VP1, VP2, and the minor polypeptide have accessible surface tyrosine residues, which may mean that these polypeptides are located predominantly at the exterior of the components. VP2 was iodinated less efficiently in virus particles than in top components when lactoperoxidase was used to iodinate, and this may indicate a conformational difference between virus particles and top components.

Acrylamide gel electrophoresis of intact

virus particles and top components. Electrophoresis of mixed DNV1 particles and top components on 3% acrylamide gels at pH 9.0 showed two well-separated bands (Fig. 6), the faster moving with twice the mobility of the slower. Purified virus particles and top components, respectively, comigrated with the bands migrating rapidly and slowly towards the anode; electron microscope observation of particles recovered from the bands detected in a natural mixture of virus and top components confirmed that virus particles migrated more rapidly. The rate of migration was directly proportional to the current applied. It also appeared that the proportion of top component to virus in partially purified preparations was 3:1 (as assessed by scanning negative photographs of Coomassie blue-stained gels), confirming earlier estimates (20). Thus, both virus and top component were negatively charged at pH 9.0, the former having twice the net charge of the latter.

Dissociation of virus particles and top components of DNV1. Experiments dissociating DNV1 particles and top components were carried out to give information about forces stabilizing virus particles and top components and to enable meaningful titration studies to be carried out (see below). The virus protein was dissociated by incubating the material with an equal volume of twice-concentrated denaturant at 30°C for 24 h. Table 3 shows a summary of the effects of a variety of denaturing conditions on the sedimentation properties.

2.5% SDS, 2.5 M NaCl, and 3 M LiCl-4 M urea failed to break down DNV particles and top components and so were not suitable for preparing protein subunits from the virus.

Treatment with either 66% acetic acid or 6 M guanidine hydrochloride-0.1 M 2-mercaptoethanol successfully dissociated top components to low-molecular-weight components (Table 3).

TABLE 2. Calculation of percentage of each polypeptide in DNV1 and DNV2 and their respective top components^a

Protein	% of protein ($\pm$ standard deviation) in:			
	DNV1		DNV2	
	Particles	Top component	Particles	Top component
VP1	11.9 $\pm$ 0.2	13.6 $\pm$ 0.1	15.2 $\pm$ 0.2	11.4 $\pm$ 0.2
VP2	28.9 $\pm$ 0.3	28.5 $\pm$ 0.3	22.7 $\pm$ 0.3	27.6 $\pm$ 0.3
VP3	22.1 $\pm$ 0.3	21.0 $\pm$ 0.3	24.1 $\pm$ 0.4	22.4 $\pm$ 0.3
VP4	37.1 $\pm$ 0.3	26.9 $\pm$ 0.5	38.2 $\pm$ 0.4	38.6 $\pm$ 0.4

^a The proteins were separated by polyacrylamide gel electrophoresis, detected by Coomassie blue staining, and scanned on a Joyce-Loebl Chromoscan with an integrator attachment.

FIG. 5. Densitometric traces of DNV2 ^{125}I -labeled polypeptides resolved by autoradiography of 10% SDS-polyacrylamide gels of virus particles (A) and top components (B) iodinated by chloramine-T after disruption by heating in SDS (i), lactoperoxidase labeling of intact virus (ii), and chloramine-T labeling of intact virus (iii). Note that the three high-molecular-weight polypeptides were radiolabeled in intact virus.

FIG. 6. Polyacrylamide gel electrophoresis of intact DNV1 particles and top components on 3% cylindrical gels. (A) Virus particles; (B) top component and virus particles; (C) top component.

A high pH had a more marked effect on virus particles than on top components. The two entities (at ≈ 1 mg/ml) were dialyzed into 0.05 N NaOH at pH 12.7 or 0.05 N NaOH-1.0 M NaCl at pH 12.3 at 4°C for 48 h. The products were then analyzed by analytical ultracentrifugation, using Schlieren optics. In low salt at high pH, the top component sedimented as a symmetrical peak with a sedimentation coefficient of 61S, suggesting that it was essentially unchanged, whereas the virus particles sedimented as three peaks with sedimentation coefficients of 9, 119, and 122S. In 1.0 M NaCl and high pH, the top component sedimented as two components with sedimentation coefficients of 2 and 47S (the latter was the major component), and the virus particle sedimented as a single peak of 12S. Thus, the top components were fairly stable to alkaline conditions up to pH 12.5, whereas under similar conditions of high pH and ionic strength, virus particles were less stable than top compo-

TABLE 3. *Dissociation of DNV particles by various denaturing solvents: effects on sedimentation coefficient^a*

Solvent	Sedimentation coefficient		Approximate $s_{20,w}$	
	Top component	Virus particle	Top component	Virus particle
2.5% SDS, pH 7.0	50S	97S	50	97
2.5% NaCl, pH 7.0	29S	63S	51	102
3 M LiCl-4 M urea, pH 7.0	16S	29S	56	90
0.05 N NaOH, pH 12.7	61S	9S, 119S, 122S	61	9, 118, 122
0.05 N NaOH-1 M NaCl, pH 12.3	2S, 47S	11S	2, 47	14
0.05 N HCl-1 M NaCl, pH 1.45	48S	95S	59	112
0.05 N HCl, pH 1.45	61S	122S	61	122 ^b
66% acetic acid	0.45	ND ^c	1	ND
6 M guanidine hydrochloride-0.1 M mercaptoethanol	0.95	ND	2	ND

^a The sedimentation coefficients of virus particles and top components in 0.01 M phosphate buffer-0.1 M NaCl, pH 7.0, are 117 and 59 respectively (8).

^b Trace amounts detected.

^c ND, Not done.

nents. The stabilities of both virus particles and top components at a particular high pH decreased with increasing ionic strength. The stability of top component was thus little affected by the titration of basic groups on the surface, which caused the particle to have a net negative charge. It is likely that some of the stability of top component arises from strong hydrophobic protein-protein interactions. The virus may be less stable than top component at a pH greater than 12 due to the ionization of the bases in the DNA.

Samples of virus particles and top component, each containing 1.2 mg/ml, were dialyzed at 4°C for 60 h into 0.05 N HCl, pH 1.45, and 0.05 N HCl-1.0 M NaCl at pH 1.45. The samples of virus particles and the corresponding samples of top component were then sedimented in pairs in the analytical ultracentrifuge. At low pH and in high salt, virus particles and top component both sedimented as single symmetrical peaks with sedimentation coefficients of 112 and 59S, respectively. Little or no dissociation had occurred in either virus particles or top component. At low pH and in low salt, top component showed some dissociation to slow-sedimenting material, but the main component was still apparently intact top component, with a sedimentation coefficient of 61S. In contrast, only a trace of intact virus particles was present in the corresponding virus particle sample. Thus, top component is stable to acidic conditions down to pH 1.45. Under similar conditions of low pH and ionic strength, virus particle was no more stable and probably less stable than top component. The stabilities of both virus particles and top component at a low pH decreased with decreasing ionic strength. The stability of top component was little affected by the titration of side-

chain carboxyl groups on the surface, which caused the particle to have a net positive charge. Again, some of the stability of top component is probably due to hydrophobic protein-protein interactions. Virus particles might be less stable than top component at pH 1.45 if the DNA was partially accessible to protons and, hence, if some of the negatively charged phosphate groups on the DNA were neutralized by protons. The pK of these phosphate groups is about 1.0, and thus they are not normally titrated. However, a pH of 1.45 might be low enough for some of these phosphate groups to be neutralized. Such neutralization might cause disrupting forces sufficient to overcome the stabilizing forces and, hence, dissociation of virus particles under conditions in which top component is stable. Alternatively, ionization of some of the bases on the DNA could make it a destabilizing influence. The effect of ionic strength again suggests that ionic forces are partly involved in destabilizing both virus particles and top component at this pH.

Continuous electrometric titration. Top components after exhaustive dialysis into 0.1 M KCl had a pH of 6.0. It was assumed that neither K⁺ nor Cl⁻ ions bound preferentially to top components, and hence this pH was taken to be the point of zero net proton charge for the purpose of interpreting the electrometric titration curve. The titration curve of top components obtained by forward-titrating samples in 0.1 M KCl with 0.1 N HCl and then back-titrating with 0.1 N NaOH is shown in Fig. 7. Top components were partially precipitated between pH 4 and 6. The titration curves were reversible between pH 2.5 and 6.0 and partly reversible, but with time-dependent changes, in the pH range 6.0 to 11.5. The acid endpoint was well defined at pH 2.5,

FIG. 7. Forward electrometric titration of DNV1 top components in 0.1 M KCl.

but the alkali endpoint had not been reached by pH 11.5. Titration curves of an equivalent volume of 0.1 M KCl were obtained, and the number of H^+ ions bound by the protein was obtained in the usual way.

A preliminary analysis of the groups titrated was made as described by Tanford (32). Seven moles of H^+ ions per 100 mol of amino acid residues were bound between pH 6.0 and 2.5. These may be assigned to side-chain carboxyl residues of aspartic acid and glutamic acid which have pK values of ≈ 4.0 . The number of histidine, lysine, and arginine residues per 100 mol of amino acid residues available for titration above pH 6.0 must therefore be seven, since at the isoionic pH (6.0) the net negative charge equals the net positive charge (32). Since 1 mol of H^+ ion per 100 mol of amino acid residues was dissociated between pH 6.0 and 7.3, presumably from histidine, a total of 6 mol of lysine and arginine residues per 100 mol of amino acid residues was available for titration. Amino acid analysis has shown that there is between 6 and 7 mol of lysine and arginine per 100 mol of amino acid residues in the protein of top components and virus particles (see Table 1 and reference 20); it is likely, therefore, that all these residues are positively charged at pH 6.0 and hence available for neutralizing negative charges on the DNA in virus particles.

Between pH 7.3 and 11.5, between four and five H^+ ions were dissociated per 100 mol of amino acid residues. The side chains titrating in this range would have been from some, but not necessarily all, of the cysteine, tyrosine, and lysine residues. The arginine residues would not be expected to titrate below pH 12. Since only 7 of the 20 aspartic acid and glutamic acid residues (20) per 100 mol of amino acid residues titrated,

13 of these residues in top component were present in the amide form. Thus, all the lysine and arginine residues in top component are titratable and hence are potentially available for neutralizing negative charges on the DNA in virus particles.

The forward titration curve of virus particles in 0.1 M KCl is shown in Fig. 8. Since virus particles had a pH of 5.7 after dialysis into 0.1 M KCl, this was taken as the point of zero net proton charge. From pH 2.5 to 5.5, virus was partially precipitated. In contrast to top components, the acid endpoint for virus particles was not reached by pH 2.5. Between pH 3.0 and 5.7, 7 mol of H^+ ion per 100 mol of amino acid residues was dissociated. For top components the corresponding value between pH 2.5 and 6.0 was also 7 mol. Between pH 2.5 and 3.0, two extra H^+ ions were apparently dissociated per 100 mol of amino acid residues in the virus. Between pH 5.7 and 3.0, therefore, the number of titratable groups of virus particles per 100 mol of amino acid residues was the same as that of top components, but below about pH 3.0 there were extra titratable groups on virus particles as compared with top components. The alkali titration curve of virus was carried out in mM KCl. Fifteen or sixteen moles of H^+ ion per 100 mol of amino acid residues was dissociated for virus particles between pH 5.7 and 11.5. The corresponding value for top components was 6 mol. Clearly, there are extra titratable groups in virus particles as compared with top components in this pH range.

Electrometric titrations were carried out under conditions which maintained top component

FIG. 8. Forward electrometric titration of DNV1 particles (—), pH 2.5 to 5.7 in 1.0 M KCl and pH 5.7 to 11.5 in 0.001 M KCl. The difference (-----) is also shown between the forward electrometric titrations of top components (Fig. 7) and virus particles.

and virus particles intact. This was confirmed by analyzing the titrated samples at both high and low pH values in the centrifuge. In all cases intact virus and top component were observed. Therefore, any difference in the titration curves between top component and virus particles cannot be due to dissociation effects.

Thus, the electrometric titration experiments on top component (Fig. 7) and virus particles (Fig. 8) showed that a larger number of hydrogen ions per 100 mol of amino acid residues was dissociated from intact virus than from intact top component in the pH range 2.5 to 11.0. The extra titratable groups of virus particles, as compared with top component, were dissociated in the pH range 6.0 to 11.0 and possibly also in the pH range 2.5 to 3.0. No extra groups were titrated from virus particles in the pH range 3.0 to 6.0, as compared with top components. These observations were not due to the different ionic strength conditions used for the titrations in the alkaline range, since the effect of increasing ionic strength would be to decrease the number of H^+ ions dissociated at high pH (34). This is contrary to the effect found. The difference in titration curve for virus particles and top component (see Fig. 8) suggested that at pH greater than 6.0, the two types of particles have different charges and hence different electrophoretic mobilities. We showed earlier that virus particles had a mobility about twice that of top component at pH 9.0. The mobility difference was consistent with virus particles' having a negative charge about twice that of top component at this pH. This is confirmed by the titration curves.

The difference in titration behavior (Fig. 8) for virus particles and top component may be ascribed to titration of the DNA bases in the virus. The four nucleotide bases of DNA provide three titratable amino groups with pK' near 4 and two titratable hydroxyl groups with pK' near 11. The fact that little or no virus DNA titrated between pH 3.0 and 6.0 but that viral DNA apparently titrated in the pH range 6.0 to 11.0 means that the titration curve for the nucleic acid in virus particles is shifted to a lower pH range compared with DNA in moderate-ionic-strength solutions and is similar to that of denatured, and hence essentially single-stranded, DNA under conditions of very high ionic strength (4). Thus, the DNA in virus particles appears from its titration curve to be in an environment equivalent to a very high ionic strength, where the net charge on the phosphate groups is almost completely screened, as would be the case if the phosphates were efficiently neutralized by counterions.

The near equivalence of the isoionic pH of the virus and top component leads to the important

conclusion that the net charge on the protein does not change when it is combined with the nucleic acid. This means that the negatively charged phosphate groups ($pK \approx 1$) on the DNA must be balanced by an equivalent number of positive counterions in the virus particle which do not come from the viral protein. In other words, the majority, if not all, of the positive charges on the viral protein are not involved in neutralizing DNA phosphates. These must be neutralized, therefore, by small counterions present within the virus particle. The electrostatic interaction between charges on the protein and DNA appears to be minimal.

A comparison of the titration curves of virus and top component shows that the protein side-chain carboxyl groups have very similar pK values in both complexes. They are probably situated on the surface of the protein capsid and are probably responsible for the solubility of the virus in aqueous solution. Similar conclusions may also be drawn concerning the arginine, histidine, and lysine side chains. These, too, are freely accessible to H^+ ions in both complexes and may therefore be situated on the outer surface of the protein capsid.

Circular dichroism studies on DNV2 particles and top components. The circular dichroic spectra of DNV1, DNV2, and the respective top components in the range 230 to 320 nm have already been reported (11). The circular dichroic spectra in the 195- to 250-nm region of virus particles and top components are shown in Fig. 9. The spectra were significantly different. There were decreases in negative dichroism of the top component between 230 and 215 nm when the protein was incorporated into the virus capsid and increases in the range 215 to 198 nm which might at first sight be interpreted as changes in the secondary structure of the protein. However, it is also possible that the changes arose from the altered environment of side-chain aromatic chromophores, such as tyrosine or tryptophan, which might have interacted with the DNA in the virus, or from altered conformation of the bases in the DNA itself. It is not possible to distinguish between these various possibilities on the basis of these spectra alone. It can be concluded, however, that there is a definite interaction between viral DNA and protein which gives rise to altered optical properties.

DISCUSSION

DNV1 and DNV2 are similar, but not identical, viruses as judged by biophysical, biochemical, and serological properties of the protein coat. This basic conclusion complements the demonstration that the viruses possess closely homologous DNAs of similar structure (13).

FIG. 9. Circular dichroism spectra at room temperature of DNV2 virus particles (-----) and top components (—).

The two viruses show typical parvovirus morphology and size (9), although no detailed structure could be determined. We were unable to confirm the deduction of Kurstak and Coté (18) that the virus contains 42 capsomeres.

The polypeptide compositions of a given DNV and its corresponding top component are identical, and both contain at least four polypeptides. The sum (265,000) of the molecular weights of the polypeptides exceeds the coding capacity of a genome with no overlapping or spliced genes—the molecular weight of the DNV genome is approximately 1.95×10^6 , and so it potentially codes for about 2×10^5 daltons of protein. Preliminary peptide mapping experiments show that the polypeptides of DNVs are interrelated (N. F. Moore and D. C. Kelly, unpublished data). Calculations show that the relative amounts of the various polypeptides are not easily accommodated in virus particles, assuming that particles containing plus and minus strands have identical polypeptide compositions. The virus contains about 60 polypeptide molecules, of which about half are the major polypeptide (VP4). The relative amounts of each polypeptide present differ considerably from the amounts calculated by Tijssen et al. (33). This probably reflects their unconventional method of calculation. Under conditions which resolve three polypeptides for adeno-associated virus particles (30), four DNV polypeptides were resolved, and so it appears that these viruses are indeed different despite similarities in the fine structures of their DNAs. Iodination experiments with both

lactoperoxidase and chloramine-T showed that only the two high-molecular-weight polypeptides and a minor polypeptide were accessible for iodination. This observation again contrasts with observations on the iodination of adeno-associated virus type 3 capsid protein (22), where all three adeno-associated virus polypeptides are iodinated. However, the observation that chloramine-T fails to iodinate the two lower-molecular-weight polypeptides does not necessarily mean that these are "core" polypeptides. It could indicate merely that in the native protein state, their tyrosine residues are inaccessible due to tertiary folding of the polypeptide chain. Omission of a reducing agent from the sample buffer failed to alter the polypeptide profile of DNA polypeptides, confirming the observation of Tijssen et al. (33), which demonstrates that disulfide bonds are not involved in the creation of the remarkably stable coat of these viruses.

Gel electrophoresis of intact virus particles at pH 9.0 showed that, in the case of DNV1 (but not DNV2, which failed to enter the gel), the virus particles could be separated from top component and indicated that the virus particles had a higher net negative charge than did the top component; this is probably attributable to the negative charge of the DNA in the virus arising from titration of the DNA bases at pH 9.0.

The conclusion that most of the DNA phosphate is neutralized by cations other than basic amino acids is supported by our earlier observation that the virus particles, but not the top component, are rich in polyamines, having a sufficient amount to neutralize 26% of the DNA (15).

The biochemical, biophysical, and serological tests described so far in this discussion have failed to show that there is any difference between the top component protein and the protein coat in situ in the virus particle for a given virus. Although we have presented indirect evidence that there is little direct interaction between the viral genome and its protein, nevertheless it is probable that interaction occurs, albeit on a limited scale, and that the interaction has a certain specificity. To determine whether a conformational difference occurred in the coat when present in top component or in part of the virus particle, circular dichroism studies were performed, and differences were detected. Analysis of the results by standard methods failed to demonstrate that the virus protein existed in a predominant secondary structural form (similar observations have been made for other single-stranded RNA viruses [11, 28, 31]). Interaction of single-stranded nucleic acid with the protein induced a marked change in optical properties of the protein, nucleic acid, or both.

The analyses of the titration data suggest that the electrostatic interactions between DNA and protein in the virus are minimal. The negative charge on the DNA appears to be completely screened by small counterions, such as polyamines. Although nonpolar interaction cannot be excluded, and indeed the differences in the circular dichroic spectra between top component and virus would support such interaction, the picture that emerges is one where the DNA is physically contained within the protein, with little direct interaction between them.

ACKNOWLEDGMENTS

We thank C. F. Rivers, M. H. Bew, M. D. Ayres, M. K. Arnold, and T. Lescott for technical assistance and R. H. Pain and R. Burgess for use of their Dichrographe III.

LITERATURE CITED

- Barwise, A. H., and I. O. Walker. 1970. Studies on the DNA of a virus from *Galleria mellonella*. FEBS Lett. 6:13-16.
- Bourguignon, G. J., P. J. Tattershall, and D. C. Wood. 1976. DNA of minute virus of mice: self priming non-permuted single stranded genome with a 5' terminal hairpin duplex. J. Virol. 20:290-306.
- Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62:315-323.
- Cox, R. A., and A. R. Peacocke. 1956. Electrometric titration of the sodium salts of deoxyribonucleic acids. III. The effect of sodium chloride. J. Chem. Soc., p. 2499-2512.
- Doty, P., and J. T. Edsall. 1951. Light scattering in protein solutions. Adv. Protein Chem. 6:35-121.
- Eggstein, M., and F. U. Kreutz. 1955. Protein determinations. Klin. Wochenschr. 33:879-889.
- Elliott, R. M., T. Lescott, and D. C. Kelly. 1977. Serological relationships of an iridescent virus (type 25) recently isolated from *Tipula* sp. with two other iridescent viruses (types 2 and 22). Virology 81:309-316.
- Fairbanks, C., T. L. Stock, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10:2606-2617.
- Gerry, H. W., T. J. Kelly, and K. I. Berns. 1973. Arrangement of nucleotide sequences in adeno-associated virus DNA. J. Mol. Biol. 79:207-225.
- Hoggan, M. D. 1971. Small DNA viruses, p. 43-79. In K. Maramorosch and E. Kurstak (ed.), Comparative virology. Academic Press, Inc., New York.
- Isenberg, H., R. I. Cotter, and W. B. Gratzer. 1971. Secondary structure and interaction of RNA and protein in a bacteriophage. Biochim. Biophys. Acta 232:184-191.
- Jones, A. S., W. Lee, and A. R. Peacocke. 1951. Determination of the phosphorous content of sodium deoxyribonucleate. J. Chem. Soc., p. 623.
- Kelly, D. C., A. H. Barwise, and I. O. Walker. 1977. DNA contained by two densovirus viruses. J. Virol. 21:396-407.
- Kelly, D. C., and H. M. Bud. 1978. Densovirus virus DNA: analysis of fine structure by electron microscopy and agarose gel electrophoresis. J. Gen. Virol. 40:33-43.
- Kelly, D. C., and R. M. Elliott. 1977. Polyamines contained by two densovirus viruses. J. Virol. 21:408-410.
- Kelly, D. C., and T. W. Tinsley. 1972. The proteins of iridescent virus types 2 and 6. J. Invertebr. Pathol. 19:273-275.
- Kurstak, E. 1972. Small DNA densovirus virus. Adv. Virus Res. 17:207-242.
- Kurstak, E., and J. R. Coté. 1969. Proposition de classification de virus de la densovirus basée sur l'étude de la structure moléculaire et des propriétés physioco-chimiques. C. R. Acad. Sci. 268:616-619.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.
- Longworth, J. R., T. W. Tinsley, A. H. Barwise, and I. O. Walker. Purification of a non-occluded virus of *Galleria mellonella*. J. Gen. Virol. 3:167-174.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
- Lubeck, M. D., and F. B. Johnson. 1977. Radiolabeling of adenovirus-associated virus external structural proteins. Virology 83:453-457.
- Mejbaum, W. 1939. Z. Phys. Chem. (Frankfurt am Main) 258:117-120.
- Meynadier, G., C. Vago, G. Plantevin, and P. Atger. 1964. Virose d'un type habituel chez le Lepidoptera *Galleria mellonella* L. Rev. Zool. Agric. Appl. 63:207-209.
- Moore, N. F., and D. C. Kelly. 1974. Sendai virus structural proteins: analysis of polypeptides linked by disulphide bonds. Intervirology 2:128-133.
- Moore, N. F., J. M. Kelley, and R. R. Wagner. 1974. Envelope proteins of vesicular stomatitis virions: accessibility to iodination. Virology 61:292-296.
- Peacocke, A. R., and I. O. Walker. 1962. The thermal denaturation of sodium deoxyribonucleate. III. Light scattering studies. J. Mol. Biol. 5:564-569.
- Piazzolla, P., V. Guantieri, C. Vovlas, and A. M. Tamburro. 1977. Circular dichroism studies of chicory yellow mottle virus. J. Gen. Virol. 37:359-372.
- Rivers, C. F., and J. F. Longworth. 1972. A non-occluded virus of *Junonia coenia* (Nymphalidae:Lepidoptera). J. Invertebr. Pathol. 20:369-370.
- Rose, J. A., J. V. Maizel, Jr., J. K. Inman, and A. J. Shatkin. 1971. Structural proteins of adenovirus-associated viruses. J. Virol. 8:766-770.
- Tamburro, A. M., V. Guantieri, P. Piazzolla, and D. Gallitelli. 1978. Conformational studies on particles of turnip yellow mosaic virus. J. Gen. Virol. 40:337-344.
- Tanford, C. 1962. The interpretation of hydrogen ion titration curves of proteins. Adv. Protein Chem. 17:70-165.
- Tijssen, P., J. van den Hurk, and E. Kurstak. 1976. Biochemical, biophysical, and biological properties of densovirus virus. I. Structural proteins. J. Virol. 17:686-691.
- Walker, I. O. 1965. The electrometric titration curves of histone and nucleohistone. J. Mol. Biol. 14:385-393.