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Biochemical, Biophysical, and Biological Properties of Densonucleosis Virus

I. Structural Proteins

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The polypeptide composition of highly purified densonucleosis virus was studied by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The viral proteins showed a different behavior in sodium dodecyl sulfate-gels in comparison with the marker proteins. Therefore, the molecular weights were estimated by analyzing the retardation of the electrophoretic mobility of these proteins in gels with increasing polyacrylamide concentrations. Four structural proteins with molecular weights of 49,000, 58,500, 69,000, and 98,000 were found, and they were designated p49, p59, p69, and p98, respectively. There are several indications that p98 is a dimer of p49. The relative quantity of the structural proteins in a virion suggests that at least p49 (accounting for ±70% of total protein mass) is a capsid protein and that there will be 12 capsomers per virion.

Densonucleosis virus (DNV) is the agent of the densonucleosis disease of *Galleria mellonella* larvae (14). This virus has many properties similar to those of the parvoviruses (9). It contains a single-stranded DNA with a molecular weight of 1.6×10^6 and, as in the case of the adeno-associated satellite viruses, the complementary DNA strands are separately encapsidated (11, 13). The size of the DNV particles were reported by different authors to be in the range of 19 to 24 nm (9). Electronmicroscope studies of the capsid of DNV showed that this virus has 42 capsomers, in contrast to the 32 capsomers for parvoviruses (10, 12). Therefore, this virus was tentatively classified into a new family, Densoviridae, placed between the Parvoviridae and Papilloviridae (10).

Different values have been reported concerning the buoyant density of infectious DNV virions in CsCl, namely 1.40 g/ml (19) and 1.44 g/ml (5). Unpublished observations from this laboratory indicate that both values are correct and that virions of both densities exist. This phenomenon has also been observed for some parvoviruses (5).

Parvoviruses, the structural proteins of which have been described, contain three structural proteins with molecular weights between 50,000 and 100,000 (6-8, 15, 16). One of these three proteins represents the main protein (about 60 protein molecules per virion) and corresponds probably to the capsid protein.

The purpose of the present study was to characterize the structural proteins of DNV and to compare their properties with those reported for the parvoviruses.

MATERIALS AND METHODS

Production and purification of DNV. *G. mellonella* larvae were injected intraperitoneally with 1 μg of virus suspended in phosphate-buffered saline. The larvae were incubated at 34 C for 6 to 8 days and stored at −20 C after their death.

The larvae were then homogenized in phosphate-buffered saline containing a few crystals of 1-phenyl-2-thiourea to prevent melanization, using a Virtis 23 mixer. Large particles were removed by centrifugation at 10,000 rpm for 10 min in an SS-34 rotor in a Sorvall RC2-B centrifuge.

Lipids were extracted from the supernatant by vigorous shaking with an equal volume of chloroform-butanol mixture (1:1), and the phases were separated by centrifugation (10 min, 10,000 rpm). The aqueous phase was then centrifuged for 1 h at 16,000 rpm.

The virus was pelleted from the supernatant by centrifugation for 2 h at 35,000 rpm in an A-321 rotor in an IEC-B60 ultracentrifuge. The sediment was suspended in 0.5 ml of 0.1 × SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate). Any unsuspended material was removed by centrifugation for 5 min at 10,000 rpm. The supernatant was then centrifuged for 2 h at 100,000 × g. The sediment was finally suspended in 0.1 × SSC and filtered through membrane filters (Millipore Corp.) of decreasing pore size (5, 1.2, and 0.45 μm).

Centrifugation in a sucrose density gradient. Linear sucrose gradients, 8 to 37% in 0.1× SSC, were prepared with a Beckman density gradient former. The virus preparation was dissolved in 5% sucrose,

0.4-ml aliquots were layered on top of these gradients, and the tubes were centrifuged for 2.75 h at 30,000 rpm at 18 C in an SB-283 swinging bucket rotor in an IEC-B60 ultracentrifuge.

After centrifugation the tubes were punctured, and the sedimentation profiles were obtained with a Beckman DU spectrophotometer equipped with an IEC flow cell. The fractions containing the virus were collected and dialyzed against several changes of 0.1× SSC at 4 C.

Isopycnic centrifugation in CsCl. CsCl (suprapur Merck AG) was dissolved in 0.1× SSC containing the virus preparation.

The density of this solution was 1.40 g/ml, as determined from the refractive index measured with an Erma refractometer. The uniformly mixed solutions were centrifuged in an SB-405 swinging bucket rotor for 25 h at 40,000 rpm in an IEC-B60 ultracentrifuge at 20 C. The gradients were recorded as described above.

Dissociation of DNV virions. Since the fraction banding at a density of 1.40 g/ml contains the complete virus (manuscript in preparation), only these virions were used in the present study.

The proteins were reduced with 1% β-mercaptoethanol and solubilized in 1% sodium dodecyl sulfate (SDS) at 95 C for 5 min in 0.5 ml of 0.01 M phosphate buffer, pH 7.1.

The sulfhydryl groups were either carboxymethylated, cyanoethylated, or oxidized. Carboxymethylation was performed by the addition of 56 mg of iodoacetamide to 1 ml of the virus sample (1.2 mg of DNV/ml).

Cyanoethylation with acrylonitrile was essentially the same as described by Seibles and Weil (17). Performic acid oxidation was performed either by the method of Hirs (4) or Geelen et al. (2).

Molecular weight estimation of the DNV protein. The technique of Weber and Osborn (21) was used for molecular weight estimation of the DNV protein. Lysozyme, chymotrypsinogen, pepsin, ovalbumin, bovin serum albumin, and β-galactosidase were used as marker proteins: these proteins were treated the same way as the viral proteins. The molecular weights were determined in polyacrylamide gels at five different acrylamide concentrations (5, 6.25, 7.50, 8.75, and 10%). The proteins were stained with 0.2% Coomassie brilliant blue R250 in methanol-water-acetic acid (50:50:7 by volume).

At each gel concentration 10 values were determined for each protein.

Retardation coefficient of DNV proteins in SDS-polyacrylamide gels. The electrophoretic mobilities of the proteins, determined at different gel concentrations for the estimation of the molecular weights of the viral proteins, were also used for the estimation of the retardation coefficients (K_R) of DNV proteins in SDS-polyacrylamide gels.

The K_R values were calculated (20) from the slopes of the straight lines produced by plotting the logarithm of the R_f of each protein against the total polyacrylamide concentration at which the observation was made (Ferguson plot).

Molecular weight estimation of DNV proteins from their retardation coefficients. There is a linear relationship between the retardation coefficient and the molecular weight of a protein (20). Using a standard curve obtained with the marker proteins, the molecular weights of the viral proteins were readily determined.

Estimation of the number of molecules of each protein in a DNV virion. The relative quantity of each type of viral protein was estimated by densitometry after separation on SDS-polyacrylamide gel and subsequent staining with 0.2% Coomassie brilliant blue R250. The densitometry was performed on a Joyce-Loebl Chromoscan MK II double-beam recording densitometer with an automatic integrator. Different known quantities of viral proteins (expressed in absorbance at 260 nm units of pure virus) have been analyzed, because protein-dye complexes may deviate from Beer's law at low or high protein concentrations (1, 3). A balancing wedge range of 3 optical density units was used, with an scanning slit of about 5 × 0.5 mm. A filter with a maximum transmission at a nominal wavelength of 500 nm was used. The image was magnified by placing a standard objective into the transmission unit. Scan expansion relative to the gel was either three or nine times.

RESULTS

Dissociation and subsequent SDS-polyacrylamide gel electrophoresis. After electrophoresis, one major protein (I), two intermediate proteins (II, III), and one minor protein (IV) were revealed (Fig. 1). The proteins had been designated provisionally as I, II, III, and IV in order of their increasing size. This pattern did not change after carboxymethylation, cyanoethylation, or oxidation of the reduced proteins.

Molecular weight estimation of the DNV proteins by the technique of Weber and Osborn. The estimates for the molecular weights of the viral proteins, obtained by the method of Weber and Osborn (21), varied considerably by changing the gel concentration (Fig. 2). This variation was particularly large for the major and minor viral proteins, producing a difference of about 10% in the estimation of the molecular weight in 5 or 10% polyacrylamide gels.

Retardation coefficients of DNV proteins and the marker proteins in SDS-polyacrylamide gels. Figure 3 depicts the "Ferguson plots" (log R_f versus gel concentration) for the reference and viral proteins derived from the data given in Fig. 2.

Molecular weight estimation of the DNV proteins from their retardation coefficients. The K_R values of the reference proteins, plotted against their molecular weights, produced a linear relationship (Fig. 4). From the K_R values of the viral proteins, the following molecular weights were obtained: 49,000 for I, 58,500 for II, 69,000 for III, and 98,000 for IV.

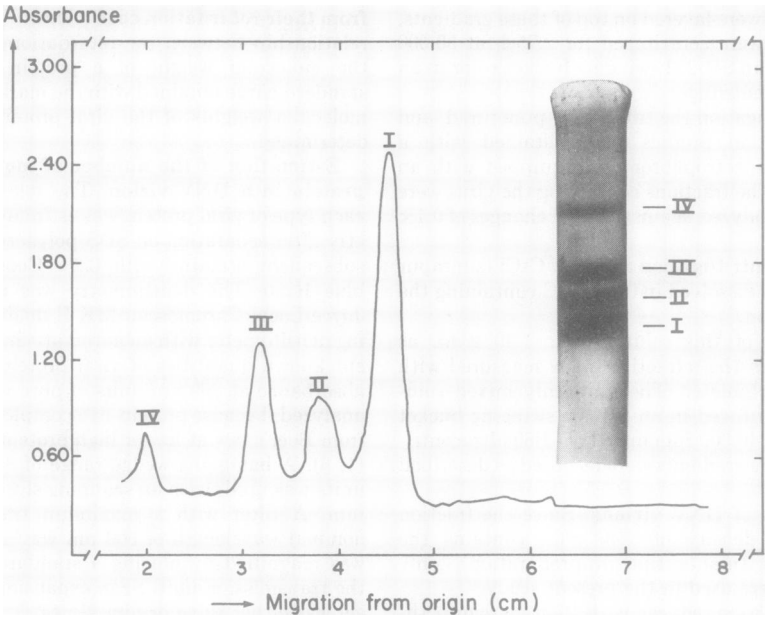

FIG. 1. *Densitometric record of the structural proteins of DNV. Settings were as indicated in Materials and Methods. The inset shows the separation of these proteins on a 7.5% SDS-polyacrylamide gel.*

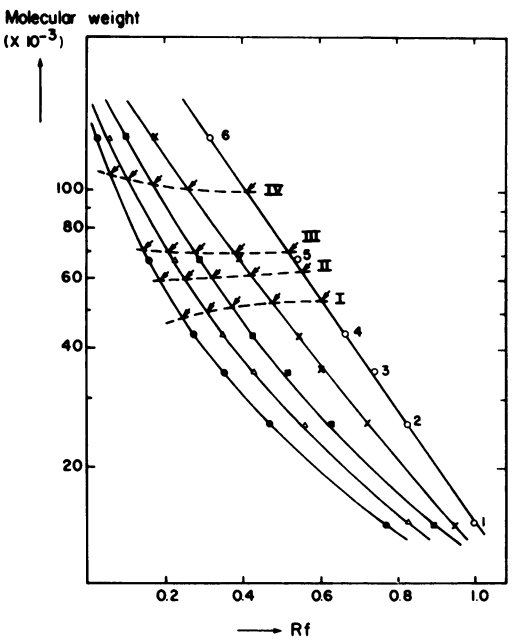

FIG. 2. *Estimation of the molecular weights of the DNV proteins by the method of Weber and Osborn at different polyacrylamide concentrations Symbols: ○, 5.0%; ×, 6.25%; ■, 7.5%; △, 8.75%; ●, 10.0%. The marker proteins are: 1 = lysozyme; 2 = chymotrypsinogen; 3 = pepsin; 4 = ovalbumin; 5 = bovine serum albumin; 6 = β-galactosidase.*

Therefore, these proteins have been designated p49, p59, p69, and p98, respectively.

Estimation of the number of molecules of each protein in the virion. Densitometry of different amounts of viral proteins, after their separation by electrophoresis on SDS-polyacrylamide gel, revealed that the net integrator counts (surface areas) plotted against the quantity of dissociated virus applied gave linear relationships (Fig. 5). The tangent of the angles *a*, *b*, and *d* are the measures of the quantity of the corresponding proteins. Thus, the relative quantity of the protein p49 is (tan a)/[(tan a) + (2 tan b) + (tan d)] = 0.69. For p59 and p69 a value of 0.15 was found, and for p98 a value of 0.01 was found.

The aggregate molecular weight of the proteins in one virion can be estimated to be 4.0×10^6, since the particle weight (5.6×10^9) and the molecular weight of the DNA (1.6×10^6) have been determined earlier (9, 11).

These data enable us to calculate the number of molecules of each protein per virion, as given in Table 1.

DISCUSSION

Electrophoresis of the dissociated DNV virions revealed the presence of four bands on SDS-polyacrylamide gels, irrespective of the way the sulfhydryl groups were blocked.

At the present, it is suspected that p98 is a dimer of p49. This suggestion is based on the following two observations. (i) In different experiments the percentage of p98 fluctuated largely (depending on salt conditions), but the

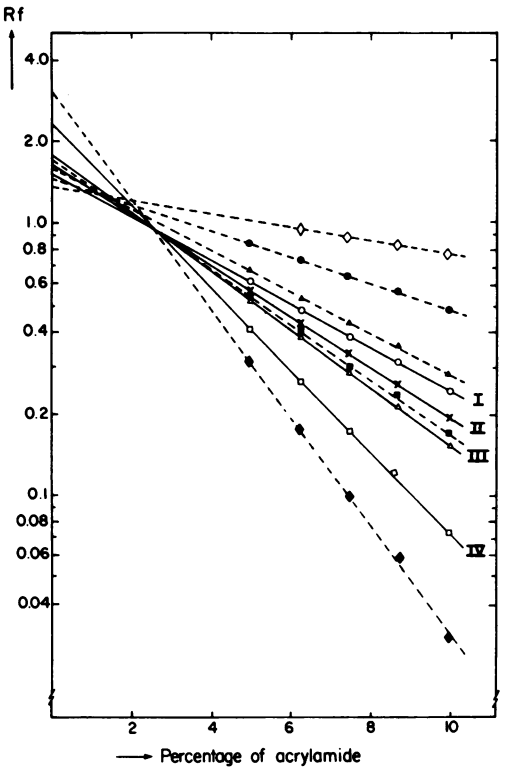

FIG. 3. *Ferguson plots of the marker and viral proteins Symbols:* ◆, *β-galactosidase;* ■, *bovine serum albumin;* ▲, *ovalbumin;* ●, *chymotrypsinogen;* ◇, *lysozyme.*

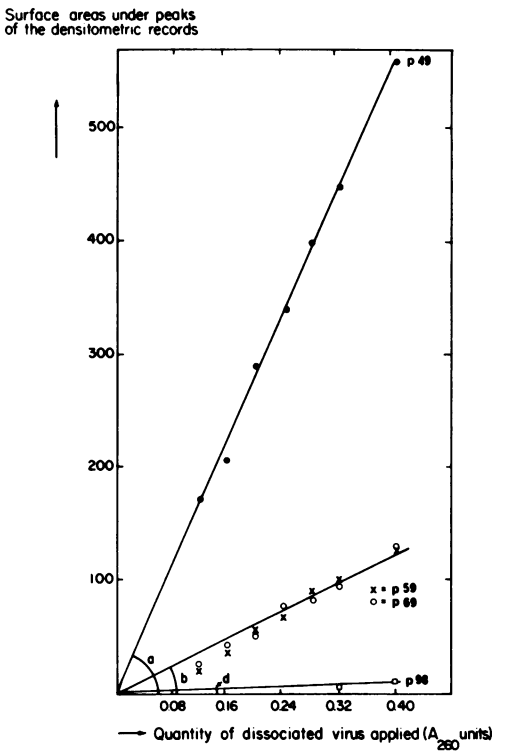

FIG. 5. *Estimation of the relative quantities of the viral proteins from their densitometric records after electrophoresis on SDS-polyacrylamide gel.*

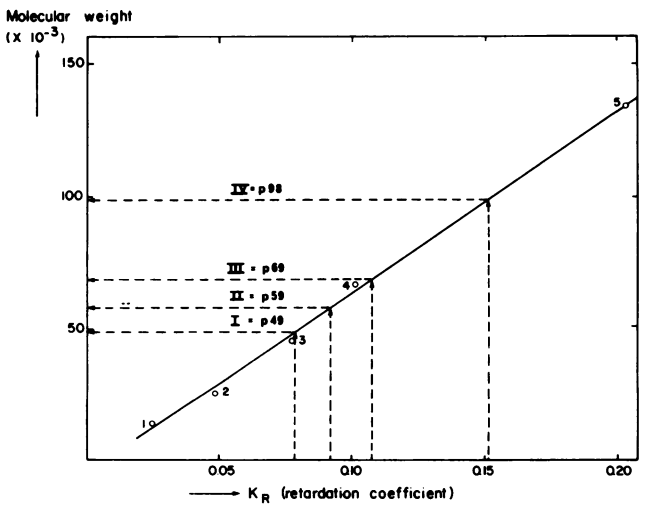

FIG. 4. *Estimation of the molecular weights of the viral proteins from their retardation coefficients. Reference proteins: 1 = lysozyme; 2 = chymotrypsinogen; 3 = ovalbumin; 4 = bovine serum albumin; 5 = β-galactosidase.*

sum of the amounts of p49 and p98 was always constant. (ii) The molecular weight of p98 is exactly double that of p49.

Since blocking the sulfhydryl groups had no effect on the viral proteins, it was expected that the proteins of the virions were not linked by disulfide bridges but by secondary forces. This conclusion was supported by the observation that the omission of β-mercaptoethanol did not influence the dissociation of the virion by SDS. In the case of adeno-associated virus type 3, the presence of a fourth band of a protein of higher molecular weight was observed occasionally on SDS-gels (7).

Tinsley and Longworth (18), in their review on parvoviruses, cited a conference presentation on structural proteins of DNV. They reported that three different proteins are found in 5% polyacrylamide gels but that four are found in 10% gel systems, a phenomenon never found in our studies. Moreover, the molecular weights they obtained for these proteins are very different from our results (72,000, 57,000, 53,000, and 46,000). The quantity of each protein in a virion was not given.

The reliability of the estimation of the molecular weights of the viral proteins by the method of Weber and Osborn (21) was tested by carrying out the electrophoresis in a series of gels of different concentrations. The experiments gave variable results for the different systems (Fig. 2), which might be due to an unusual shape or an anomalous SDS-binding of the viral proteins.

TABLE 1. *Calculation of the number of molecules of each protein per virion*

Protein	%	Protein mass ($\times 10^{-6}$)	mol wt	No. of polypeptides
p49	69	2.76	49,000	56.7
p59	15	0.60	58,500	10.2
p69	15	0.60	69,000	8.6
p98	1	0.05	98,000	0.5

By plotting the logarithm of the relative migration against the gel concentration (Ferguson plots), straight lines were obtained for all proteins, the slope of which increased with increasing molecular weight. Extrapolation of these lines resulted in two intersections (Fig. 3), one intersection of the viral proteins and β-galactosidase and one intersection of the marker proteins. Why the DNV proteins behave as β-galactosidase remains a problem. The fact that the marker proteins have another intersection than the viral proteins explains why, by the method of Weber and Osborn (21), at different gel concentrations unlike molecular weights for the same protein were obtained.

The Ferguson plots enabled us to estimate the molecular weights of the DNV proteins. A comparison of the molecular weights as estimated by the two different methods is given in Table 2.

It is of interest to compare these results with the molecular weights available to date on the structural proteins of parvoviruses. A comparison is given in Table 3.

The estimation of the number of protein molecules per virion can be considered as reliable since the viral proteins were studied over a wide range of concentration. In this method the only assumption made is that all viral protein are stained uniformly.

The mass of the main protein of DNV and other parvoviruses (either I or II) is equivalent to about 60 protein molecules per virion. This, in turn, could mean that each virion consists of 12 capsomers (pentamers), as is the case for φX174, and not of 32 or 42 capsomers estimated by electron microscopy for parvoviruses (5).

It has been reported for almost all the parvoviruses that the aggregate molecular weights of their proteins exceeds the coding capacity of the viral genome.

The resemblance of the protein structure of DNV and of the parvoviruses is striking and suggests that DNV might belong to this family.

Further studies on the localization of each

TABLE 2. *Comparison of two different methods for the estimation of the molecular weight of the viral proteins*

Protein	mol wt determined by method of Weber and Osborn (21)					mol wt estimation from retardation coefficients
	5.00[a]	6.25	7.50	8.75	10.00	
p49	52,500	52,000	50,000	49,000	48,500	49,000
p59	62,000	61,500	59,500	60,000	59,500	58,500
p69	70,000	70,000	70,000	70,000	70,000	69,000
p98	100,000	100,000	105,000	106,000	108,000	98,000

[a] Concentration (percent) of acrylamide.

TABLE 3. *Comparison of the molecular weights of the structural proteins of the parvoviruses and of DNV*[a]

Protein	DNV	Kilham rat virus (16)	H-1 (8)	AAV (15)	AAV-3 (7)	Haden (6)
I	49,000	52,000	56,000	62,000	65,900	66,800
II	58,500	62,000	72,000	73,000	79,300	76,750
III	69,000	72,000	92,000	87,000	91,000	85,500
IV	98,000	–	–	–	±[b]	–

[a] The underlined figures indicate the main protein for each virus, representing about 70 to 80% of the total protein mass. The molecular weights of the DNV proteins were determined from their retardation coefficients; all other values were obtained by the method of Weber and Osborn (21). AAV, Adeno-associated virus.

[b] ±, Sometimes a fourth band was obtained.

protein within the virion, the serological relationships amongst them, and their amino acid composition are presently in progress.

ACKNOWLEDGMENTS

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