Characterization of N-Polyhedrin of Two Baculovirus Strains Pathogenic for *Orgyia pseudotsugata*†

George F. Rohrmann‡

ABSTRACT: N-polyhedrin of inclusion bodies of two nucleopolyhedrosis viruses of *Orgyia pseudotsugata* was characterized. Alkali-dissolved N-polyhedrin from both virus strains was of similar size and consisted of a 12S molecule of 209 000 daltons. Eight subunits of approximately 26 000 daltons were found to form the 12S molecules. N-polyhedrin from both viruses showed two main antigens by immunodiffusion. The subunits appear to possess one antigen and, upon formation of the 12S molecule, a new antigen is created. Both the subunit and 12S antigens from the two virus strains were shown to be antigenically related. The 12S molecule of both viruses also appears to possess a minor antigen unique to each virus.

Nucleopolyhedrosis and granulosis virus (Baculovirus subgroups A and B) infections of insects result in the production of crystalline protein inclusion bodies in which mature viruses are occluded. The proteins of the crystalline bodies (virions excluded) have been named "N-polyhedrin" and "granulin", respectively (Summers and Egawa, 1973). Crystals produced by both types of viruses indicate close structural relationships. Bergold (1963a) and Harrap (1972), using electron microscopy, concluded the crystals were composed of a cubic lattice. Shapiro and Ignoffo (1971) and Faust et al. (1973) found that crystals from two nucleopolyhedrosis viruses contain high concentrations of inorganic cations and silicon. The crystals dissociate under alkaline conditions into molecules of approximately 12 S (Bergold, 1947; Kozlov et al., 1969; Scott et al., 1971; Longworth et al., 1972; Harrap, 1972) with molecular weights estimated at 276 000–378 000 daltons (Bergold, 1963b). Longworth et al. (1972) found in the granulosis virus of *Pieris brassicae* that these molecules are capable of forming higher order polymers. Dissociation in vivo appears to be facilitated by the presence of an alkaline protease which degrades the inclusion body protein (Kozlov et al., 1975a; Eppstein et al., 1975; Eppstein and Thoma, 1975; Summers and Smith, 1975). Recently it has been shown that the basic subunit of the proteins is a molecule of 28 000–29 000 daltons (Kozlov et al., 1975b; Eppstein et al., 1975; Summers and Smith, 1975). In the granulosis virus of *Trichoplusia ni*, this subunit is phosphorylated (Summers and Smith, 1975). Amino acid analyses of N-polyhedrin and granulin from a number of viruses indicate they are high in dicarboxylic amino acids or their amides (Kozlov et al., 1975b; Eppstein et al., 1975; Cibulsky, 1975; Summers and Smith, 1975). N-polyhedrins of three lepidopteran viruses have isoelectric points of pH 5.3–5.7 (Bergold and Schramm, 1942), and two granulin components from *Pieris brassicae* have isoelectric points of pH 3.5 and 5.8 (Longworth et al., 1972). Antisera to inclusion body protein indicate the presence of at least two antigens by immunodiffusion (Krywienczyk and Bergold, 1961; Tanada and Watanabe, 1971; Longworth et al., 1972; Scott and Young, 1973). N-polyhedrins produced by a number of viruses infecting lepidoptera were found to share two antigens and possess one unique antigen by immunodiffusion (Krywienczyk and Bergold, 1961).

Douglas fir tussock moth (*Orgyia pseudotsugata*) larvae are subject to infection by two nucleopolyhedrosis viruses. The bundle virus (BV) strain is characterized by the presence of bundles of 5–15 virus rods, whereas, in the single-rod (SV) strain, virions occur individually in the polyhedra (Hughes and Addison, 1970; Hughes, 1972). This report describes physical, biochemical, and immunochemical investigations conducted on the polyhedrin of BV and SV strains to determine the size, structure, and antigenicity of the protein components and to examine the relationship of the polyhedrin from the inclusion bodies of the two viruses.

Materials and Methods

Protein. Inclusion bodies were produced and purified by the methods of Martignoni et al. (1968). Purity of the polyhedron suspensions was confirmed by biological assay in *O. pseudotsugata* larvae, by electron microscopy (Hughes and Addison, 1970), and by dark-field microscopy procedure, with alkaline hydrocolloid (Martignoni, 1972). Polyhedrin was prepared by the methods of Summers and Smith (1975). Inclusion bodies at 5 mg/mL were heat treated at 70 °C for 20 min to inactivate the protease and then dissolved by adding 0.1 volume of 1 M Na_2CO_3–0.5 M NaCl. The inclusion bodies were allowed to dissolve at room temperature for 10 min. The solution was then cooled to 4 °C and cleared at 120 000*g* for 45 min at 4 °C to eliminate virions and undissolved inclusion bodies or their fragments. The supernatant was dialyzed against 0.01 M Tris,[1] pH 8.9, overnight at 4 °C. This preparation was used or concentrated by lyophilization.

Antisera. Antisera to polyhedrin were made by injecting rabbits with alkali-dissolved polyhedrin at a concentration of 3–5 mg im at weekly intervals for 4–5 weeks. One month after the final injection, 1 mg of protein was injected iv and the rabbits were bled 1–2 weeks later.

Antisera to the polyhedrin subunits were produced with lyophilized protein dissolved in 6 M guanidine hydrochloride

† From the Forestry Sciences Laboratory, Pacific Northwest Forest and Range Experiment Station, Forest Service, United States Department of Agriculture, Corvallis, Oregon 97331. *Received October 21, 1976.* Supported in part by funding (to Dr. M. E. Martignoni) from the Expanded Douglas-Fir Tussock Moth Research and Development Program of the United States Department of Agriculture.

‡ Current address: Department of Agricultural Chemistry, Oregon State University, Corvallis, Oregon 97331.

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; im, intramuscularly; iv, intravenously; PAGE, polyacrylamide gel electrophoresis.

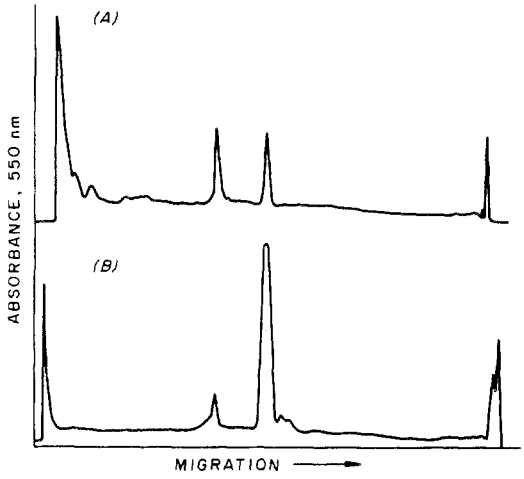

FIGURE 1: Alkali-dissolved BV polyhedrin electrophoresed in sodium dodecyl sulfate-PAGE. (A) Protein was made 1% sodium dodecyl sulfate and heated at 100 °C for 5 min before electrophoresing. (B) Protein was made 1% sodium dodecyl sulfate and 0.1% 2-mercaptoethanol and heated at 100 °C for 5 min before electrophoresing. Protein (7.6 μg) on 10% gels, 6 cm in length, was electrophoresed for 45 min at 3 mA/gel.

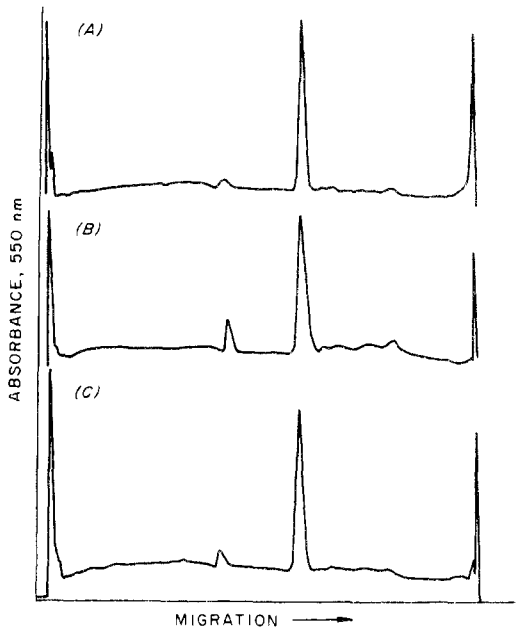

FIGURE 2: Comparison of reduced SV and BV polyhedrin. (A) BV polyhedrin (0.7 μg); (B) SV polyhedrin (0.7 μg); (C) BV and SV polyhedrin mixed (0.35 μg each). Protein was made 1% sodium dodecyl sulfate and 0.1% 2-mercaptoethanol and heated at 100 °C for 5 min and electrophoresed on 6-cm 10% sodium dodecyl sulfate gels at 3 mA/gel for 45 min.

to a concentration of 10 mg/mL. Shortly before injection, 2-mercaptoethanol was added to the solution to a concentration of 0.05–0.1%, and it was heated at 100 °C for 5 min. Every 2 weeks, 2.5–5.0 mg was injected subcutaneously for a total of 4–5 injections. The animals were bled 2 weeks after the final injection.

The antigens for the initial im injections were in complete Freund's adjuvant and the subsequent im injections were in incomplete Freund's adjuvant. Adjuvant was not used for the iv injections.

Analytical Ultracentrifugation. Measurements were made at 20 °C using a Spinco Model E analytical ultracentrifuge.[2] Sedimentation velocity and low-speed sedimentation equilibrium runs were done in 0.01 M Tris (pH 8.9). The molecular weight of alkali-dissolved polyhedrin was determined using low-speed sedimentation equilibrium at 5206 rpm for 25 h with a protein concentration of 0.3 mg/mL. Molecular weights of subunits were determined using high-speed sedimentation equilibrium runs in 6 M guanidine hydrochloride with 0.1% 2-mercaptoethanol at 44 000 rpm for 27 h with protein concentrations of 0.4–0.8 mg/mL. An apparent partial specific volume of 0.733 was used for the protein in 6 M guanidine hydrochloride (Thomas and Edelstein, 1971).

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was done following the procedures of Maizel (1967). No sample or spacer gel was used, and 5- to 25-μL samples in 20% sucrose were layered on the surface of the resolving gel. Bovine serum albumin (68 000), bovine λ globulin light (23 500) and heavy (50 000) chains, pepsin (34 500), and lysozyme (14 500) were used as molecular weight markers. Gels were stained with Coomassie blue R-250 and scanned at 550 nm with a Gilford 2400 linear transport accessory on a Beckman DU spectrophotometer.

Double Immunodiffusion. Double immunodiffusion using 1% agarose on microscope slides was done according to the methods of Crowle (1961); 10 μL of reactants was normally added per well.

Amino Acid Analysis. Amino acid analysis was conducted using the method of Moore and Stein (1963). The protein hydrolysate was chromatographed on a Beckman Spinco Model 120 B amino acid analyzer. Tryptophan was determined spectrophotometrically (Edelhoch, 1967).

Results

Physical Characterization. The main component of alkali-dissolved polyhedrin of both strains is a molecule with a $s_{20,w}$ of 11.6–12.0 as shown by velocity sedimentation. The SV also contained a molecule of 18.8 S similar to that reported by Longworth et al. (1972) and considered a dimer of the 12S molecule. In a mixture of equal concentrations of BV and SV polyhedrin solutions, the smaller components sedimented as one 11.6S peak. The molecular weight of the BV 12S molecule was determined by low-speed equilibrium sedimentation to be 209 000. Alkali-dissolved BV polyhedrin heated at 100 °C for 5 min in 1% sodium dodecyl sulfate remained near the origin of sodium dodecyl sulfate-PAGE gels with a small amount of two lower molecular weight components migrating into the gel (Figure 1A). Upon reduction with 2-mercaptoethanol and heating at 100 °C for 5 min in 1% sodium dodecyl sulfate, the 12S molecule was dissociated into one major low-molecular-weight component (Figure 1B). The minor component of higher molecular weight is also normally present. SV polyhedrin behaved similarly; when mixed, the peaks of both samples comigrated, indicating similar size (Figure 2). Using sodium dodecyl sulfate-PAGE and marker proteins, the molecular weight of the major component was determined to be approximately 26 000 ± 1000, indicating the 209 000 molecular weight, 12S molecule is composed of eight subunits. The minor component comigrated with bovine serum albumin in sodium dodecyl sulfate PAGE, indicating a molecular weight near 68 000.

Amino Acid Analysis. The amino acid composition of polyhedrin from both virus strains is similar (Table I). The pol-

[2] The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the United States Department of Agriculture of any product or service to the exclusion of others which may be suitable.

TABLE I: Amino Acid Composition of Bundle Virus (BV) and Single-Rod Virus (SV) Polyhedrin.

Amino Acid	BV		SV	
	Mol %	No. of Residues[a]	Mol %	No. of Residues[a]
Lys	8.5	18	7.6	15
His	3.8	7	3.0	6
Arg	7.4	12	9.0	15
Asx	11.0	25	10.6	24
Thr	3.7	10	3.6	9
Ser	3.4	10	4.2	12
Glx	11.5	23	11.8	24
Pro	5.8	15	6.4	17
Gly	2.6	12	2.6	12
Ala	2.6	10	3.4	12
Val	6.5	17	6.0	16
Met	2.4	5	1.9	4
Ile	5.7	13	4.4	10
Leu	7.0	16	7.3	17
Tyr	6.5	10	7.8	13
Phe	7.3	13	7.0	12
½-Cys[b]	1.3	3	1.3	3
Trp	2.6	4	2.4	3

[a] Number of residues based on a molecular weight of 26 000. [b] Cystine determined as cysteic acid.

yhedrin from both viruses is high in dicarboxylic amino acids or their derivatives and contains three half-cystine residues.

Antigen Characterization. Antisera were made to heat-treated alkali-dissolved molecules containing predominantly the 12S protein and its breakdown products (Figure 1A), and to polyhedrin subunits made by reducing and denaturing polyhedrin in 2-mercaptoethanol and 6 M guanidine hydrochloride. High-speed equilibrium sedimentation indicated that both BV and SV polyhedrin in the 6 M guanidine hydrochloride–0.1% 2-mercaptoethanol solution are a homogeneous population of molecules of approximately 29 500 ± 500 daltons.

Heat-treated and untreated antigens both reacted with antisera made to heat-treated antigen, indicating heat treatment does not affect the major polyhedrin antigens (Figure 3).

Antisera to alkali-dissolved polyhedrin produce two major precipitin lines in immunodiffusion (Figure 4). The more rapidly diffusing molecules are likely subunits produced from the breakdown of the 12S molecules. The more slowly migrating molecule is likely the 12S protein. Antisera to subunits produced one major precipitin line which appeared related to the subunit component of both BV and SV alkali-dissolved preparations (Figure 4). A minor precipitin line was also frequently observed migrating slightly slower than the subunits and may be the 68 000-dalton molecule observed in PAGE scans (Figures 1 and 2) and is possibly of viral or cellular origin.

The BV and SV major precipitin lines fuse, indicating the subunits and 12S antigens of both polyhedrins are related. The 12S molecule displays minor spur formation for both viruses, indicating the presence of a unique viral antigen on the 12S molecule. These reactions can be interpreted as follows: The polyhedrin subunits have one antigen common to SV and BV; upon forming the 12-S molecule, two new antigens are created: one common to both BV and SV and the other unique to each virus. Antisera to subunits are weak, but, if used in sufficiently high concentration, they precipitated both components in alkali-dissolved polyhedrin preparations (Figure 5). This indicates that the subunit antigen is present on the 12S molecule. Therefore the 12S molecule likely possesses at least three antigens including the subunit antigen, the common 12S antigen, and the unique 12S antigen.

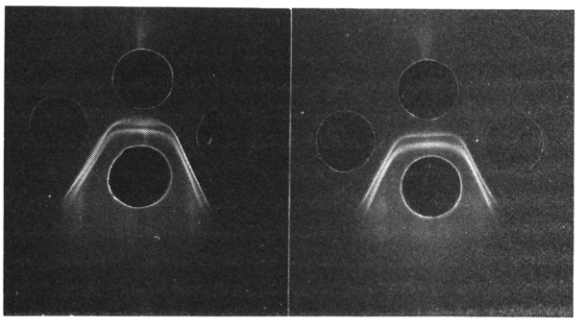

FIGURE 3: Effect of heat treatment on antigenicity. Left template: (bottom well) BV antisera to heat treated alkali-dissolved polyhedrin; (top well) BV alkali-dissolved polyhedrin (not heat treated); (left and right wells) alkali-dissolved polyhedrin preheated at 70 °C to inactivate the protease. Right template: same as left template only with SV antigens and antisera. All antigens at 1 mg/mL; reaction was at 4 °C for 24 h.

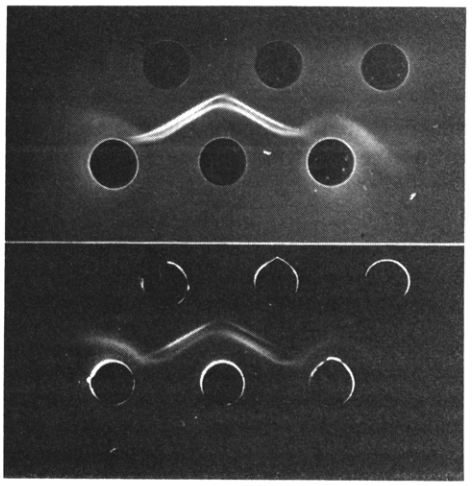

FIGURE 4: Relationship of BV and SV polyhedrin. Upper template (top row antigens left to right): BV polyhedrin, SV polyhedrin, and BV polyhedrin. Bottom row left to right: antisera to BV subunits, BV alkali-dissolved polyhedrin, and BV subunits. Lower template (top row) same as upper template; (bottom row) same as upper templates, except antisera to SV components used. All antigens, 1 mg/mL; subunit antisera was concentrated three times by lyophilization; all antigens were alkali-dissolved heat-treated polyhedrin. Reaction was at 4 °C for 72 h.

Discussion

These studies indicate that eight polyhedrin subunits may be linked by disulfide bonds to form the 12S molecule. If all subunits are bonded by disulfide bonds to neighbors, as in the corners of a cube, at least three half-cystine residues per subunit would be necessary. If we assume a polyhedrin subunit of 26 000–29 000 daltons, two to four half-cystine residues can be calculated from the amino acid analyses from a number of investigators (Kozlov et al., 1975b; Cibulsky, 1975; Summers and Smith, 1975). Amino acid analysis indicates the presence of high concentrations of dicarboxylic amino acids or their amides. The proteins have a low isoelectric point (Bergold and Schramm, 1942; Longworth et al., 1972), indicating they are predominantly in the dicarboxylic form. The presence of phosphate groups on the protein (Summers and Smith, 1975)

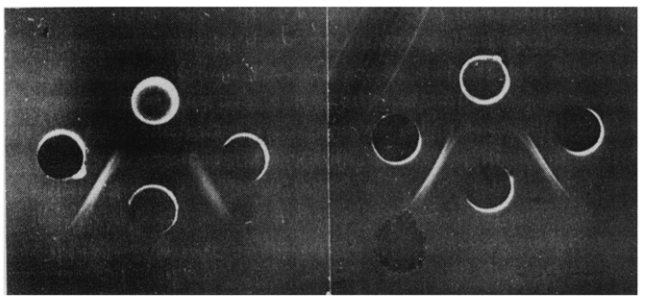

FIGURE 5: Absorption of alkali-dissolved polyhedrin antigens by antisera made to subunits. Left template: (bottom well) antisera to alkali-dissoloved BV polyhedrin; (top well) alkali-dissolved BV polyhedrin + antisera to BV subunits; (left and right wells) alkali-dissolved BV polyhedrin. Right template: same as left template, except SV antigen and antisera used. Antigen concentrations were 0.5 mg/mL. The absorbed solution was prepared by mixing antisera to subunits concentrated three times by lyophilization with 0.25 volume of antigen (0.5 mg/mL). The well was loaded four times to bring the antigen concentration up to 0.5 mg/mL and the reaction was at 4 °C for 24 h.

could further contribute to a low isoelectric point. In the insect cell, it is likely the negatively charged polyhedrin molecules readily attract and are neutralized by inorganic cations, thereby allowing crystallization to occur. This could account for the high concentration of inorganic cations reported by Shapiro and Ignoffo (1971) and Faust et al. (1973).

The two viruses studied differ by as much as 8% in guanine plus cytocine concentration (G. Rohrmann, unpublished data) and therefore probably differ significantly at the genetic level. Antigenic studies on the two viruses indicate the polyhedrin genes have been conserved over an extended period of viral evolution. It is possible that the slight variation observed is in a region not directly involved in inter- or intramolecular bonding. Since the main function of polyhedrin appears to be stabilizing the virus outside the host, it is evident that this role has been performed adequately and therefore there has been little pressure for its modification.

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