Physical and Chemical Properties of *Trichoplusia ni* Granulosis Virus Granulin

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The protein solubilized from the proteinic crystalline structure surrounding the granulosis virus of *Trichoplusia ni* by use of a carbonate buffer (pH 10.7) gives a major component, as analyzed by ultracentrifugation, with a molecular weight of 180,000. This protein has heterogeneous subunit structure as demonstrated by estimates of molecular weights by use of gel electrophoresis, amino-, and carboxy-terminal analyses, and peptide mapping of enzyme digests of the protein. The amino acid composition shows that the protein is acidic with a high percentage of amino acids with hydrophobic side groups. Optical rotatory dispersion studies reveal the presence of β -structure in the protein complex. The conversion of the β -structure to α -helix with sodium lauryl sulfate and to a random coil state with strong alkaline treatment are observed.

Occluded insect viruses commonly referred to as granulosis viruses (or capsules) (GV) and polyhedrosis viruses (NPV) have large proteinic crystalline structures surrounding the enveloped virion or bundles of nucleocapsids. This protein crystal is known to be extremely stable against solubilization by many solvents at neutral pH values and physiological conditions, and highly resistant against the action of proteolytic enzymes (4, 5). Early studies on protein dissociated from the crystalline structure by weak alkali showed the proteins had sedimentation constants and estimated molecular weights of 11.5S (275,000), 12.7S (336,000), and 12.8S (378,000) for NPV from three different insect species, and 11.8S (300,000) for a GV (6). Recently, it was suggested on the basis of immunological studies that the apparently homogeneous protein in the crystalline structures of a NPV and a GV contain at least two different proteins (22, 33, 40). However, the antigenic specificity of a protein or the determinants thereof may change, dependent upon the state of aggregation of a protein (42). Therefore, during the initial characterization of granulin and polyhedrin, a class of proteins which demonstrate extreme solubility properties, immunological studies must be correlated with physical and chemical techniques to properly characterize the protein. Early studies have reported that the macromolecular structure of the larger protein subunits (approximately 12S) could be dissociated by stronger alkaline treatment into

subunits which are homogeneous with respect to molecular weights (5).

In our previous reports (13, 19), the solubilizing effects of various solvents on the proteinic crystalline structure of a granulosis virus of *Trichoplusia ni* were followed by kinetic and morphological studies in an attempt to evaluate the nature of intermolecular binding forces which contribute to the construction and stability of the structure. In this report the physicochemical characteristics of the protein solubilized from this structure are studied in greater detail.

The relationship of a protein crystal to a virus, occluding it as such, is an interesting and, in many aspects, unique association of such structures with viruses characteristic of the occluded insect viruses. In the past such descriptions as "inclusion-body protein," "crystal protein," "proteinic crystal," "capsule protein," and "polyhedral protein" have been used to describe this type of protein structure and its relationship to the virus. In addition to being perhaps misleading, it is difficult to use such expressions as now applied to our biochemical and future biophysical studies. Herein and hereafter this protein will be described as "granulin" for the proteinic crystal of granulosis viruses and "polyhedrin" for the nuclear polyhedrosis viruses.

MATERIALS AND METHODS

Preparation of samples. Occluded virus was iso-

lated from infected larvae by a combination of differential and sucrose gradient centrifugations (37). The occluded virus was further purified by washing three times with 0.02 M sodium acetate buffer (pH 5.1) containing 0.05 M sodium chloride and 0.1% (wt/vol) sodium lauryl sulfate (SLS), and then was washed three times with water, using resuspension, stirring at room temperature for 15 min, and centrifuging at $20,000 \times g$ for 15 min.

The granulin of the occluded virus thus purified was dissolved by use of a 0.07 M sodium carbonate buffer (pH 10.7) containing 0.05 M sodium chloride at a ratio of 5 mg of occluded virus per ml of solvent for 1.5 to 2.0 h at room temperature. The solubilized preparation was then centrifuged at $100,000 \times g$ for 30 min, or layered on top of and centrifuged through 10 to 40% (wt/vol) sucrose gradients after centrifugation for 25 min at 25,000 rev/min by use of a SW41 rotor. The $100,000 \times g$ supernatant or sample portion from the top of the sucrose gradient was collected and extensively dialyzed against large volumes of water at 4 C. The dialysate was used as granulin and is described as preparation A (prepn A).

Occluded virus purified as described for prepn A was concentrated by centrifugation at $20,000 \times g$ for 15 min and lyophilized. The lyophilized capsules were solubilized with the carbonate solution as described for prepn A. The granulin lyophilized once was recovered as the $100,000 \times g$ supernatant fluid or from the top of the 10 to 40% sucrose gradient after centrifugation and designated as preparation B (prepn B). Granulin lyophilized once recovered and described as prepn B was dialyzed against deionized water and again lyophilized for storage. The granulin preparation lyophilized twice was solubilized in the carbonate solution as previously described and designated as preparation C (prepn C).

Because of the extremely insoluble nature of granulin, in certain studies prepn A (nonlyophilized) or B (lyophilized once) was treated with 0.3 N NaOH at 100 C for 5 min and then cooled. This method was employed for a comparison of an extremely harsh treatment of the protein to other denaturing conditions employed in these studies. The pH of the solution was adjusted to 5.0 with dilute acetic acid and stored overnight at 0 C. The granulin thus recovered as a precipitate is described as preparation D (prepn D).

Amino acid analyses. The protein samples were hydrolyzed with constant-boiling-point, aqueous HCl (6 N) at 105 C for 20, 48, or 72 h. The amount of each amino acid in the hydrolysate was determined by automatic amino acid analyzer (JLC-6AH, Jeol Co., or Beckman Model 120C). The amounts of serine, threonine, and methionine were obtained by extrapolation of the values to zero time of hydrolysis. The amounts of the other amino acids were obtained from the hydrolysis which gave a maximum yield of the amino acid. The content of cystine and cysteine was obtained from cysteic acid content of the protein which had been oxidized with performic acid (41). Tryptophan content was calculated from the ratio of tryptophan to tyrosine estimated from the UV absorption spectrum in 0.1 N sodium hydroxide (16).

Peptide maps. Protein samples were digested with

trypsin or thermolysin in 1% ammonium bicarbonate at 37 C for 12 h. The substrate concentration was 10 mg/ml, with a final substrate-to-enzyme ratio of 50 to 1. Half of the enzyme was added at the beginning of the incubation, and the other half was added after 5 h of incubation. The hydrolysate was applied on Whatmann 3 MM filter paper and subjected to electrophoresis at pH 6.5 (10% pyridine-4% acetic acid) for the first dimension (4 KV, 40 min) and at pH 1.9 (8% acetic acid-2% formic acid) for the second dimension (3 kV, 30 min). Positions of the peptides on the paper were detected with 1% ninhydrin-0.1% cadmium acetate.

Analytical ultracentrifugation. A Spinco model E ultracentrifuge (Beckman Instrument Corp.) was used for the study of sedimentation velocity. Experiments were performed with double-sector cells having a 12-mm light path and with a schlieren optical system. Prepn A dissolved in 0.05 M sodium carbonate buffer (pH 10.5) at a concentration of 8 mg/ml was centrifuged at 15 C at an operating speed of 59,870 rev/min. The schlieren patterns were photographed between 3 to 35 min after the rotor had reached the operating speed. For the calculation of $s_{20,w}$ the partial specific volume of the protein was taken to be 0.737 ml/g, as estimated from the amino acid composition. The viscosity and the specific gravity of the solvent at 15 C was 1.1420 centipoise and 1.00413 g/ml, respectively.

Gel electrophoresis. Polyacrylamide gel (acrylamide: *N,N'*-methylenebisacrylamide = 73:2) containing 0.05 M sodium carbonate buffer (pH 10.5) and 0.1% SLS was used for the separation of protein components and also for the estimation of molecular weights (34). The same carbonate buffer containing 0.1% SLS was used as the electrode buffers. The gel concentration was 7.5% for routine studies, but 4 and 6% gels were also used in the experiments for Ferguson plots. Acrylamide was polymerized with 0.15% *N,N,N',N'*-tetramethylethylenediamine and 0.05% ammonium persulfate. For the estimation of molecular weights, bovine serum albumin (molecular weight 68,000), ovalbumin (45,000), pepsin (35,000), trypsin (24,000), lysozyme (14,300), beef pancreatic RNase (13,900), cytochrome *c* (11,700), and insulin (6,000) were used as standards.

N-terminal amino acid analysis. 1-fluoro-2,4-dinitrobenzene was recrystallized from ether. Ether was freed of peroxidized materials by treatment with aluminum oxide (Woelm-basic, M. Woelm, Eschwege, Germany) and redistilled before use. The protein samples were incubated at room temperature for 3 h in 60% ethanol containing 0.5% sodium bicarbonate and 5% 1-fluoro-2,4-dinitrobenzene (29). After the incubation, most of the dinitrophenylated (DNP) protein remained in solution. The reaction mixture was extracted with ether while it was alkaline or until no color remained in the ether phase, slightly acidified with hydrochloric acid, and again extracted three times with ether. The resulting aqueous phase and the precipitate were stored at 0 C overnight to insure acid precipitation of the DNP protein. The precipitate was collected by centrifugation and lyophilized. Concentrated formic acid was added (0.2 ml) to facilitate solution of the precipitate, a portion of which re-

mained insoluble in 6 N HCl. HCl (6 N) was then added, and hydrolysis was maintained at 100 C for 12 h. In some experiments hydrolysis was carried out for only 2 or 4 h to detect labile DNP-amino acids. The hydrolysate was diluted with 5 vol of water and extracted with ether. The ether phase was extracted with 0.1 N HCl and dried in vacuo. Contaminating dinitrobenzene was removed by sublimation by use of Mill's apparatus. The resulting DNP-amino acids were identified by two-dimensional paper chromatography (15) or by two-dimensional, thin-layer chromatography (10). The quantity of each DNP-amino acid separated on the chromatograms was determined by measuring UV absorption of 0.1% sodium bicarbonate eluate of the spot at 360 nm and a comparison with DNP standards. The value was corrected for the degradation of DNP-amino acid during the acid hydrolysis (28). For the detection of water-soluble DNP-amino acids, one-dimensional paper chromatography of the material in the water phase was carried out by use of the solvent system for the second dimension of Levy's system (21). For the detection of DNP-proline, acid hydrolysis of DNP-protein was carried out in concentrated hydrochloric acid at 100 C for 2 and 4 h (32).

C-terminal amino acid analyses. Anhydrous hydrazine was prepared from 80% hydrazine hydrate by distillation with toluene over calcium oxide (20). Hydrazine sulfate was recrystallized from water and dried at 140 C. Benzaldehyde was redistilled under nitrogen. The lyophilized protein was incubated at 60 C for 16 h with anhydrous hydrazine containing 1 M hydrazine sulfate (3, 8). After the incubation, the reaction mixture was dried in vacuo over concentrated sulfuric acid and the resulting dried material was dissolved in water and treated with benzaldehyde (2, 8). The free amino acids recovered in the water phase were analyzed by the automatic amino acid analyzer. The value obtained was corrected for the degradation of amino acids during hydrazinolysis (8).

Detection of sugars by gas chromatography. The lyophilized sample was treated in 5% methanolic-HCl (wt/wt) at 100 C for 3 h. The methanolysate was extracted with petroleum ether, treated with a small amount of Amberlite IR4B (OH⁻), and evaporated to dryness. The recovered material was trimethylsilylated in 4 vol of pyridine, 2 vol of hexamethyl disilazane, and 1 vol of trimethylchlorosilane (39, 43). The resulting trimethylsilyl derivatives of sugars were analyzed by gas chromatography (GC-4BM, Hitachi) using a column of 5% Ucon LB 550X on Gas Chrom CLH (80 to 100 mesh) (2 mm by 2 m) and a column temperature of 195 C. Xylose was added to the sample as an internal standard for the quantification of sugars.

Colorimetric determinations. Protein was measured by the Lowry method (23) using bovine serum albumin as the standard. Free α -amino groups were measured by the ninhydrin method (24) using leucine as the standard. Hexose was measured by the anthrone method (31) using glucose as the standard. RNA was measured as ribose by the orcinol reaction (11) using bacterial RNA as the standard. Phosphorus was determined by the method of Fiske and Subbarow (14).

Optical rotatory dispersion. Optical rotation was measured in the wave length range of 200 to 300 nm with a JASCO ORD/UV-5 apparatus equipped with a cell of 1-mm light path. The protein was dissolved at a concentration of 200 μ g/ml in various solvents containing sodium carbonate buffer (final concentration 0.05 M, pH 10.5). Mean residual molecular weight was calculated to be 136 from the result of amino acid analysis of the protein. The refractive index values of the solvents in UV region were substituted by the values determined in daylight with a refractometer (Bausch and Lomb).

Radioactive tracer incorporation. Protein was recovered from occluded virus which had replicated in cells in the presence of ³²P, the intent of the experiments of which was to prepare highly labeled virus DNA. Three days after infection of fourth instar *Trichoplusia ni* larvae, 100 μ Ci of ³²P-disodium phosphate per day was per os injected into each larva for a period of 6 to 7 days. The microinjection procedure as described by Paschke, Lowe, and Giese (27) was employed.

RESULTS

A large quantity of colorimetric-reacting and ³²P-associated material could still be removed from occluded virus which was purified by sucrose density gradient centrifugation. Most of this was removed by washing the occluded virus preparation with an acetate buffer (pH 5.1) containing 0.1% SLS (Fig. 1). It was shown previously by electron microscopy that the occluded virus is very stable and the internal and surface crystal structure was not notably affected by the SLS-acetate treatment (13, 19). As shown in Fig. 1, about 9% of the total protein and, when ³²P-labeled occluded virus was used, about 80% of the radioactivity were removed by a combination of all washing procedures. When ribose was determined by the orcinol reaction using RNA as the standard, the amount of orcinol-positive material was 0.35% of the dry weight. This orcinol-reacting substance was not released from the protein preparation by RNase or DNase digestion, but nearly all was released by a combination of all washing procedures.

A considerable quantity of ³²P could also be removed by the washing procedure shown and described in Fig. 1. However, more than 80% of the ³²P radioactivity remaining with the SLS-washed, occluded virus which had been labeled in vivo with ³²P, remained in the granulin solubilized with 0.03 M sodium carbonate buffer at pH 10.5. When a suspension of the ³²P-labeled occluded virus in 0.05 M sodium acetate buffer (pH 5.1) containing 0.14 M NaCl and 0.3% SLS was treated with a mixture of phenol and cresol (3:1, vol/vol) containing 0.1% 8-hydroxyquinoline, less than 10% of the radioactivity was extracted into the aqueous phase.

FIG. 1. Washing procedure of the occluded virus. Symbols: — protein; ---- orcinol positive; — ³²P. Values indicate the amount of protein, orcinol-positive material, and ³²P solubilized from the occluded virus by repeated washing as described in Materials and Methods. The first to the third washings were with 0.1% sodium dodecyl sulfate-0.05 M NaCl-0.02 M sodium acetate buffer (pH 5.1). The fourth to the sixth washings were with deionized water. For each washing occluded virus was suspended, stirred at room temperature for 20 min, and centrifuged at 4,000 × g for 20 min. The points shown for each washing should be totaled to obtain the final amount removed and represent only the amount removed for the individual treatment.

About 5% of the radioactivity in the occluded virus was extractable into chloroform-methanol (2:1, vol/vol) after the solubilization at pH 10.5 and precipitation at pH 5.1. However (as compared to controls), the total amount of phosphorus associated with the protein fraction was released into the supernatant fluid after the treatment of granulin with 0.3 N NaOH at 100 C for 5 min followed by acid precipitation of the protein. The solubilized granulin obtained from capsules after the extensive washing procedure was colorless at neutral or acid pH values and had a slight straw color at alkaline pH values.

The total amino acid composition of granulin (Table 1) shows that the solubilized protein is predominantly acidic with a large percentage of amino acids with hydrophobic side groups. Analyses of individual, separated polypeptides were not determined because the extreme insolubility and aggregation properties of the polypeptides in the preparation did not allow for their individual separation. The UV absorption spectrum of the protein in 0.1 N sodium hydroxide had absorbance maxima at 289 nm and 283 nm. From this spectrum it was calculated that the tryptophan-to-tyrosine ratio was

0.33. A comparison of the amino acid compositions of nonlyophilized granulin (prepn A) to that of granulin treated with 0.3 N NaOH and 100 C for 5 min (prepn D) shows that degradation of amino acids did not take place to a significant extent during treatment of the protein with 0.3 N NaOH at 100 C, except that about a 30% decrease of the amount of threonine and of cystine plus cysteine was observed as well as a significant decrease in lysine. The increase of glycine is very likely due to the conversion of threonine to glycine during the alkaline treatment. High pH and heat were used as an extreme condition for such a comparison in an attempt to completely denature all polypeptide aggregates since other conditions only did so partially.

Other than amino acids, granulin contained saccharides (about 1.0% of the dry weight as hexose as determined by anthrone reaction). Gas chromatographical analyses of the sugars revealed glucose (0.3% of the dry weight), galactose (0.2%), mannose (0.04%), and ribose (0.02%).

Analytical ultracentrifugation pattern of prepn A shows three peaks: $s_{20,w} = 3.45$ (broad

TABLE 1. Amino acid composition of granulin^a

Amino Acid	Mol %	
	Prepn A	Prepn D
Lys	5.4	4.7
His	2.5	2.3
Arg	6.3	6.7
Asp + AspNH ₂	12.4	11.9
Thr	5.8	3.9
Ser	3.4	3.1
Glu + GluNH ₂	12.8	12.6
Pro	6.5	7.1
Gly	4.2	5.7
Ala	4.4	4.7
Val	6.3	6.6
Met	2.7	2.2
Ileu	5.8	5.8
Leu	8.3	8.5
Tyr	4.3	5.1
Phe	5.8	6.2
Cys + ½ Cys	1.7	1.1
Try	1.4	1.6

^a The protein was hydrolyzed for 20, 48, and 72 h with constant-boiling-point, aqueous HCl at 105 C and corrected for the degradation of amino acids during the hydrolysis. For the determination of Cys + ½ Cys, the protein was oxidized with performic acid before acid hydrolysis. Try was determined from Try/Tyr ratio calculated from the UV absorption spectrum of the protein in 0.1 N NaOH. Prepn A, Nonlyophilized protein. Prepn D, Granulin treated with 0.3 M NaOH and heated at 100 C for 5 min for a comparison of the stability and/or degradation of the protein.

peak), 7.2S (main peak), and 15.8S (small peak) (Fig. 2). The main peak has a calculated molecular weight of approximately 180,000.

Two-dimensional paper electrophoretograms of granulin (prepn A or D) digested with trypsin and thermolysin are shown in Fig. 3. These experiments were conducted in an attempt to provide further experimental data for the complexity and/or number of polypeptides in granulin, as well as to establish an experimental system for comparisons with granulin and polypeptides from other occluded viruses. When lyophilized protein (prepn B or C) was used, the enzyme digestion was always incomplete, leaving a large amount of material at the origin of the paper electrophoretograms. Therefore, only nonlyophilized protein could be used for these studies. The trypsin digest of nonlyophilized granulin produced 22 ninhydrin-positive peptides: 5 acidic, 8 basic, and 9 neutral. It was suspected that, because of possible self-aggregation and the insoluble property of the protein, a significant portion of the protein was not available for digestion by trypsin. Therefore, for comparison with the trypsin digest, the protein was treated with 0.3 M NaOH and heated at 100 C for 5 min and the preparation was neutralized and dialyzed as described for prep D. Digestion of prep D with thermolysin resulted in about 40 peptides: 16 acidic, 16 basic, and 8 or 9 neutral. One of the acidic peptides in tryptic digest of prep A migrated toward the anode at pH 6.5, but did not electrophoretically migrate at pH 1.9. This indicates that the peptide has a

FIG. 2. Analytical sedimentation analysis of granulin. Solubilized nonlyophilized protein was in 0.05 M sodium carbonate buffer (pH 10.5) at a concentration of 8 mg/ml and centrifuged at 15 C. An operating speed of 59,870 rev/min for 35 min was used with a Spinco model E ultracentrifuge equipped with double-sector cells having a 12-mm light path and a schlieren optical system. Arrow shows the direction of sedimentation.

FIG. 3. Two-dimensional paper electrophoretograms of enzyme digests of granulin. (a), Tryptic digest of prep A (nonlyophilized). (b), Thermolysin digest of prep D (0.3 M NaOH, heated at 100 C for 5 min, see Materials and Methods). The electrophoresis was carried out on a Whatman 3 MM filter paper at pH 6.5 (10% pyridine-4% acetic acid) for the first dimension (4 kV, 40 min) and at pH 1.9 (8% acetic acid-2% formic acid) for the second dimension (2 kV, 30 min). In panel A of this figure the eight basic peptides appear on the upper portion of the electrophoretogram and the five acidic ones appear on the lower portion. The nine neutral peptides are observed intermediate to these. The same relative relationship of acidic basic and neutral peptides is shown in panel B. Substrate concentration was 10 mg/ml, with a final substrate-to-enzyme ratio of 50 to 1. The reaction was carried out according to the detailed procedure in Materials and Methods. Spots were visualized with 1% ninhydrin-0.1% cadmium acetate. (a) indicates strongly acidic peptide. O and Y show that the spots were orange or yellow, respectively. Dark spots indicate that the spot gave fluorescence under UV illumination.

strong negative charge. One peptide with similar mobility was detected as a yellow spot on the electrophoretogram of thermolysin digest of prepn A or C. Neither of these peptides was detected when prepn D was used as the substrate.

Table 2 presents the results of N- and C-terminal amino acid analyses of the protein. Both termini show marked heterogeneity with four major termini present. When prepn A was used, the recovery of N-terminal amino acids was always less than from that of prepn D or lyophilized protein. N-terminal threonine was detected only in prepn D. On the other hand, dinitrophenylation of ϵ -amino groups of lysine in prepn A was almost quantitative. When prepn C (lyophilized twice) was used, the recovery of α -DNP amino acids was so low that meaningful results were not consistently obtained.

The separation of the protein components and estimation of their molecular weights were carried out also by electrophoresis in polyacrylamide gel containing SLS (34). The electrophoresis resulted in several bands due to the preparation or the pretreatment, or both. In the system used, the mobility of the standard protein markers exhibited a linear relationship with the

logarithm of their molecular weights in the molecular weight region of 6,000 to 66,000 (Fig. 4). The extrapolation of this straight line to the higher-molecular-weight region was not valid because, as determined, the free mobility (Y intercept in Ferguson plot; Neville, 1971) of high-molecular-weight substances showed considerable deviation from those of low-molecular-weight substances (molecular weight 6,000 to 40,000), which had similar free mobility. This deviation indicates differences in the ratio of the effective charge to the frictional coefficient between two groups of SLS-protein complexes. Together with the sigmoidal nature of the relationship between the logarithm of molecular weight and relative mobility (26), this difference in free mobility will result in large underestimations of the molecular weights in the high-molecular-weight region. Considering the measuring error and the scattering of the points with the standard proteins, the molecular weights of the substances in each band in the molecular weight region of 6,000 to 66,000 can be estimated with the greatest degree of reliability (Fig. 4).

The relative mobility of all the bands observed in the gels in Fig. 4 are also shown in Fig. 5. Although not apparent by observation of the

TABLE 2. N- and C-terminal analyses of granulin^a

Amino Acid	N-termini				C-termini		
	Mol %		No. of residues found per 180,000 daltons		Amino Acid	Mol %	No. of residues found per 180,000 daltons
	Prepn D	Prepn A	Prepn D	Prepn A			Prepn A
Asp	33	29	4.9	1.9	Gly	31	5.1
Gly	25	24	3.7	1.5	Lys	14	2.4
Ser	18	18	2.7	1.2	Leu	12	2.1
Thr	10	0	1.4	0	Arg	12	2.0
Met	8	15	1.2	0.9	Glu	9	1.5
Ala	6	14	1.0	0.9	Ser	6	1.0
			+)—	+)—	Ala	6	0.9
					Leu	5	0.8
			14.9	6.4	His	5	0.8
							+)—
							16.6

^a N-terminal analyses were carried out by dinitrophenylation of the protein, acid hydrolysis of the DNP-protein and the two-dimensional, thin-layer chromatography (first dimension, upper layer of toluene-pyridine-ethylene chlorohydrin-0.8 N NH₄OH [100:30:60:60]; second dimension, chloroform-benzylalcohol-acetic acid [70:30:3]) of the DNP-amino acids recovered from the hydrolysate. The DNP-amino acids separated on thin-layer chromatography were eluted with 1% sodium bicarbonate, and the quantity was determined by measuring optical density at 360 nm. The values obtained were corrected for the degradation of DNP-amino acids during the acid hydrolysis. C-termini were analyzed by hydrazinolysis with anhydrous hydrazine in the presence of 1 M hydrazine sulfate at 100 C for 16 h. The hydrazinolysate was treated with benzaldehyde, and then analyzed by automatic amino acid analyzer. The values obtained were corrected for the degradation of amino acids during the hydrazinolysis.

FIG. 4. Standard line for the estimation of molecular weight by gel electrophoresis, relative mobilities of the components in once-lyophilized protein (prepn B), and estimated molecular weights. The electrophoresis of standard proteins and that of prepn B pretreated in various conditions as shown in Fig. 5(A)-(E) were carried out simultaneously by use of a 7.5% polyacrylamide containing 0.1% SLS and 0.05 M sodium carbonate buffer (pH 10.5), and at 5 mA/tube. The relative mobilities of the standard proteins, expressed relative to the mobility of insulin, are plotted against the logarithm of their molecular weight. Dotted lines show $\pm$ 5% of the standard line (solid line) with respect to molecular weight (upper). The mean relative mobility of each band in the sample is shown with the limit of measuring errors (middle). The scale is the same to that of the abscissa for the standard line. (a), (b), (c), and (d) show relative positions of the bands indicated in Fig. 5. The molecular weights of each of the components were calculated from the above data with the limit of errors (lower scale).

scanning patterns, the three bands represented by the region (d) in Fig. 4 and 5 were visually the predominant bands of protein in the gels. Although possibly there is considerable error in reporting reliable estimates of molecular weights for those high-molecular-weight bands of protein, parallel migration and comigration with gamma globulin (subunit molecular weight 160,000) and β -galactosidase (130,000) showed that these proteins had molecular weights in the 160,000 to 200,000 range and very likely represented the major band of protein (180,000) observed in the ultracentrifuge (Fig. 3).

It is notable (Fig. 5) that the relative positions of the bands are the most important to focus upon, and that the height of the peaks do not represent true quantitative differences in

quantity of protein in each of the bands. We experimentally determined this by use of a Gilford scanning spectrophotometer and by showing that recorder response fell off exponentially with an increase of protein concentration in the band of protein. It was not possible to obtain labeled protein with radioactive amino acid precursors because all attempts to do so resulted in insect virus protein with a very low specific activity. Therefore, quantification by that means was not possible.

Since the physical and chemical properties of this protein are relatively unknown, a variety of solubilization techniques have been investigated (13). It was of interest to observe the effects of some of these solvent systems and denaturation techniques on the protein preparation.

When prepn B (lyophilized once) was applied without pretreatment, the major bands of the high-molecular-weight material were in the vicinity of band (d) (Fig. 5). The protein in this band appeared to be disaggregated into those of slightly smaller molecular weight [those bands immediately below (d) and lower molecular weight] by various pretreatments. Band (a) was observed to become more predominant only when the sample was pretreated with 2% SDS (or higher) or with strong alkali at elevated temperature. Bands designated as (a) and (b) migrated faster than the marker dye. Treatment of the sample with mercaptoethanol followed by treatment with iodoacetamide did not affect the banding pattern markedly. Addition of urea did not alter the pattern essentially except in some cases bands appeared close to the top of the gel, probably due to artifactual disulfide bridge formations or nonspecific exchanges of disulfide bonds in the presence of urea under alkaline conditions. Only when the protein was heated in 0.3 N NaOH at 100 C for 5 min did bands (a) and (b) become predominant with the concomitant loss of proteins in region (d) and other regions of the gel. The mobility of the main band, band (d), of prepn C (protein lyophilized twice) without pretreatment was similar to that of prepn B (lyophilized once) but was more predominant. The conversion of the protein in this and adjacent bands in prepn C to the faster migrating components did not significantly increase even using pretreatment with 2% SDS. Therefore, lyophilization can have a significant effect upon the disaggregation of the subunit structure of protein in the (d) region.

The UV rotatory dispersion property of the protein is shown in Fig. 6. This was included to show the physical state of the protein of the preparation as used for gel electrophoresis and

FIG. 5. Gel electrophoresis of granulin exposed to various conditions (see Fig. 4). (A), Prepn B (once-lyophilized protein) without pretreatment. (B)-(E), Prepn B with various pretreatments with SLS. Treatments: (B), 0.01%, 20 C, 3 h; (C), 0.1%, 20 C, 3 h; (D), 2%, 20 C, 2 h; (E), 2%, 45 C, 16 h. (F), Prepn C (twice-lyophilized protein) without pretreatment. (G), Prepn C with pretreatment, 2% SLS, 45 C, 16 h. (H) and (I), Protein solubilized from the occluded virus with 4 M guanidine HCl (pH 8.5). (H), Without pretreatment; (I), with 4 M guanidine-HCl, 2% SLS, 20 C, 3 h. (J)-(M), Prepn A (nonlyophilized protein) treated with various conditions. (J), 2% SLS, 45 C, 30 min. (K), 1 N NH_4OH , 100 C, 5 min. (L), 0.3 N NaOH, 100 C, 3 min. (M), 0.3 N NaOH, 100 C, 5 min. Gel (7.5%) containing 0.05 M sodium carbonate buffer (pH 10.5) containing 0.1% SLS was used. After electrophoresis (5 mA/gel), the gels were stained by Coomassie blue and the banding patterns of the stained gels were recorded with a Gilford scanning spectrophotometer using a wave length of 550 nm. (e), Top of the gels; (f), bottom of the gels. Each gel pattern represents a total of 200 μg of proteins loaded per gel.

cannot be used to evaluate the physical state of individual polypeptides. The curve with prepn A shows a broad trough at 227 nm with a positive shoulder at 203 nm. This pattern can be interpreted as that of a mixture of β -structure (12, 18, 30) and α -helix. With the addition of SLS to a concentration of 2%, the minimum was shifted to 233 nm, which is characteristic of α -helical configuration, and the shoulder at 203 nm disappeared. An apparent increase of rotation was also observed. SLS (0.1%) brought about only a slight conversion. The curve with prepn D exhibited a minimum at 207 nm and a negative shoulder around 230 nm, indicating that the strong alkaline treatment converted the conformation of the protein predominantly to random coil structures. Although difficult to evaluate in a heterogeneous mixture, this information may allow some insight to the interpretation of gel banding patterns at some future time.

DISCUSSION

The molecular weight of the major component of granulin detected as dissolved in dilute alkaline saline was estimated from sedimenta-

tion velocity to be 180,000. This is a value which agrees with the size of the lattice spacing of the crystalline structure, 7 to 8 nm (39), measured by electron microscopy (assuming a globular protein). Most studies report a sedimentation coefficient in the 12 to 13S range. However, our study provides an *S* value similar to that obtained from NPV polyhedrosis of *Bombyx mori* (35). On the other hand, gel electrophoresis of the protein in the presence of SLS and additional denaturing conditions exhibited a variety of bands and banding patterns. The molecular weight of the protein in the band (d), which migrates slightly slower than β -galactosidase standards, if calculated by the extrapolation of the standard line appears to be about half of 180,000. But the comparison with the standard shows this value to be largely underestimated because of faster free mobility demonstrated by the Ferguson plot for this band, and also because of the sigmoidal nature of the standard line (26). The protein or proteins in the vicinity of band (d) is very likely the main component observed in the sedimentation analysis. The hydrophobic nature proposed for the surface of the particle (13) could be responsible

FIG. 6. Optical rotatory dispersion (ORD) of granulin. The ORD of the protein solution (200 $\mu\text{g/ml}$) in 0.01 M sodium carbonate buffer (pH 10.5) was measured using a cell of 1-mm light path. R' , Reduced mean residual rotation. Each curve is mean of three determinations. (1), Prepn A, nonlyophilized protein solubilized with 0.03 M Na_2CO_3 + 0.05 M NaCl; (2), prepn A treated with 2% SLS; (3), prepn A treated with 0.3 M NaOH, heated at 100 C for 5 min, cooled, and neutralized.

for the higher affinity for SLS (25) and thus for the higher free mobility of the protein-SLS complex. Using only the results obtained for this study it is presently premature to provide additional speculation or reasons for the additional high-molecular-weight bands of slightly lower molecular weight as compared to band (d).

The interconversions of the banding patterns due to the various pretreatments indicate that the protein is constructed of subunits, and that stepwise disaggregations of the subunits take place resulting in the liberation of the faster migrating components. The estimated molecular weight of the protein in each of the bands indicated, unlike earlier assumptions on the

homogenous subunit structure of granulin and polyhedrin (4, 5), that the structure of the protein crystal is constructed of more than two kinds of polypeptides. For example, the two fastest moving bands, (a) and (b), with molecular weights of $7,100 \pm 500$ and $10,100 \pm 1,000$, respectively, are not likely in a monomer-dimer relationship. Band (a) appears predominant only when the protein was pretreated in strong alkali or in high concentration of SLS, whereas band (b) appears with 0.1% SLS treatment. Therefore, the binding force involved in the binding of the component in band (a) is stronger than that of the component in band (b). Since 2-mercaptoethanol and iodoacetamide did not affect the banding patterns, there is likely to be no contribution of disulfide bonds for the aggregation of subunits. The intermolecular binding forms are enhanced by lyophilization of the protein as can be seen from the difficulty in disaggregation of polypeptides of nonlyophilized (prepn A) to once-lyophilized (prepn B) and twice-lyophilized protein (prepn C). This increasing resistance against disaggregation as a result of lyophilization seems to indicate that there is a large contribution of the binding forces of Vander Waal which is strengthened by removal of bound water by lyophilization. The use of 0.3 N NaOH and heating at 100 C was utilized as an extreme condition to disaggregate the protein structure. Although it has been known that conversion of arginine to ornithine, of threonine and serine to glycine, degradation of cystine and cysteine, and also cleavage of peptide bonds take place in vigorous alkaline conditions, the treatment with 0.3 N NaOH at 100 C for 5 min does not cause such degradations to a considerable extent except about 30% decrease of each of threonine and cysteine plus cystine. The increase in tyrosine is inexplicable. The results of the determinations of N-termini show that this alkali treatment does not cause extensive and random cleavage of peptide bonds and thus introduce major artifacts which could interfere with the analysis.

The complexity of the two-dimensional paper electrophoretograms of the trypsin digest of the protein, when the approximate molecular weight of the polypeptides and the content of lysine and arginine are taken into consideration, support the heterogeneity supposition of the polypeptides contained in granulin. The heterogeneity of peptides from the trypsin digest, however, do not support the speculation that there are more than four major polypeptides, if one assumes the monomer molecular weight to range from 7,000 to 12,000 or 15,000.

The results of N- and C-terminal analyses

also confirm the presence of heterogeneous polypeptides. The data seem to indicate that approximately 30, 25, 18, and 10% of the polypeptides in the material have termini of $\text{H}_2\text{N}\cdot\text{Asp}\text{---Gly}\cdot\text{COOH}$, $\text{H}_2\text{N}\cdot\text{Gly}\text{---Lys}\cdot\text{COOH}$, $\text{H}_2\text{N}\cdot\text{Ser}\text{---Leu}\cdot\text{COOH}$, and $\text{H}_2\text{N}\cdot\text{Thr}\text{---Arg}\cdot\text{COOH}$, respectively. The differences between nonlyophilized, twice-lyophilized (prepn C), and once-lyophilized but NaOH-heated and denatured granulin (prepn D) in the recovery of N-terminal amino acids as α -DNP derivatives, and the result that dinitrophenylation of α -amino group of nonlyophilized granulin (prepn A) was incomplete while the modification of ϵ -amino group of lysine was almost quantitative, suggest that some of the α -amino groups of the native protein particle are in nonreactive or in reagent unaccessible state. The reaction of the protein with β -naphthoquinone-4-sulfonic acid, which has milder reactivity than 1-fluoro-2,4-dinitrophenol with amino groups (unpublished data) shows that one amino group per about 8,000 daltons of the protein does not react with the reagent, but becomes reactive by the alkaline treatment. Some studies using protein titration curves also showed that some dissociable groups with pK values around 8 or 9, presumable α -amino groups, which are in nonionizable or masked states in the native protein are converted to ionizable or exposed states by the alkaline treatment.

It is not clear at this time whether the nonreactivity of α -amino groups is due to chemical modifications or to steric protection like hydrophobic or hydrogen-bonded shell structures. The latter, however, seems to be more probable when we take into consideration that (i) the recovery of α -DNP amino acids from the lyophilized preparation was extremely low; (ii) that lyophilization results in a greater resistance against disaggregation and also against the action of proteolytic enzymes; (iii) that some residues other than α -amino groups are also in nonionizable states and also become ionizable by the strong alkaline treatment; and (iv) that the amino acid composition shows a high percentage of hydrophobic amino acids. If the α -amino groups were chemically modified, such modification should be alkali labile.

Optical rotatory dispersion and IR studies (unpublished) revealed the presence of β -structure in the native protein, which can be converted to α -helix by the treatment with SLS. The presence of such protein structure in a mixture of polypeptides is difficult to evaluate at this time. However, it is possible some polypeptides in the β -configuration may con-

tribute to the structural stability of the protein crystal. When the difficult properties of self-aggregation of the individual polypeptides are overcome, the structural role of this protein configuration can be better studied. The maximal conversion by SLS coincided with the predominance of lower-molecular-weight proteins, particularly band (a) in the gel electrophoresis. This may provide additional evidence for the presence and role of intermolecular β -structures. It is conceivable that β -structure, with side groups of amino acids protruding perpendicularly to the axis of polypeptide backbone, and also to the direction of the interpolypeptidyl hydrogen bonds, is favorable to the formation of hydrophobic bonds between hydrocarbon side chains of the amino acids of the neighboring polypeptides. It has been reported that the binding of four subunits of thyroxine-binding prealbumin is unusually stable and the protein has β -structure between subunits (9). It is also conceivable that SLS, which cleaves hydrophobic bonds by binding with nonpolar groups of the protein, also cleaves intermolecular hydrogen bonds by electrostatic repulsion between the sulfate groups, and that the cleavage of the intermolecular hydrogen is followed by spontaneous formation of intramolecular hydrogen bonds to form an α -helix. The treatment of the protein with strong alkali, on the other hand, resulted in random coil structure accompanied by maximal disaggregation of the subunits. This conversion may be due partly to racemization of amino acids in the polypeptide chain by the alkaline treatment (7), resulting in rearrangement of side chains in a way which is less favorable to the hydrophobic associations.

The origin and the characteristics of phosphate and sugars found in the protein preparation are not clear. It is possible that the phosphate is covalently bound to one of the polypeptides which gives rise to the acidic peptide on the peptide maps; this is presently being checked by additional studies. In addition, there was a discrepancy between the amount of ribose determined colorimetrically (0.35%) and that determined by gas chromatography (0.02%). It is not clear whether there is the presence of pentose other than that of ribose or if there is a form of ribose which is not derivatized for gas chromatography like ribose found in pyrimidine nucleotides of RNA. It has been reported that there is RNA in proteinic crystalline structures of occluded insect viruses on the basis of that orcinol-reacting substance. Phosphate and nucleotide bases are extractable from the occluded viruses (1, 17). Interestingly, this RNA was not easily hydrolyzed into mononu-

cleotides by alkali. The RNase resistance of the orcinol-reacting substance and the inability to extract a large portion of the phosphate, as shown in this study, tend to suggest, although not definitely, that they are not derived from RNA. It is possible that the sugars are present only as contaminants.

The unusual stability of the proteinic crystalline structure of the granulosis virus as described above is of interest not only from the viewpoint of its origin, the protection of the virion, and involvement in the virus infection process, but also from the aspect of the rigid self-assembly of the protein subunits. Further study will elucidate the more detailed mechanism of subunit interactions.

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