Characterization and Serology of the Matrix Protein from a Nuclear-Polyhedrosis Virus of *Trichoplusia ni* before and after Degradation by an Endogenous Proteinase

By DEBORAH A. EPPSTEIN* and JOHN A. THOMA

Department of Chemistry, University of Arkansas, Fayetteville, AR 72701, U.S.A.

(Received 1 February 1977)

The intact matrix protein from a nuclear-polyhedrosis virus of the cabbage looper (*Trichoplusia ni*), isolated after inhibition of an endogenous serine-type proteinase, was further purified by molecular-sieve chromatography. The matrix protein was associated with carbohydrate moieties, and the carbohydrate content was determined for the two major peptides isolated after proteolysis by the endogenous proteinase. The association-dissociation interactions of the intact and proteinase-hydrolysed monomer units were characterized at high and low pH. At pH 1.9, proteinase-degraded matrix protein dissociated into two different peptide fractions, FI and FII. Fraction FII, a single peptide of 9400 daltons, comprised one-third of the monomer unit of 28 000 daltons. At pH 9.5, the degraded peptides were tightly associated in units equivalent to the intact monomer. These monomer equivalents associated to form a series of interconverting aggregates. The predominant aggregate sedimented at 11 S and had a mol.wt. $\geq 200\,000$. Two non-cross-reacting antigens were present in the aggregate mixture. The presence of these two antigens does not reflect the presence of two different matrix proteins; rather, the expression of the antigens correlates with the degree of aggregation of the matrix protein.

Nuclear-polyhedrosis viruses of the baculovirus genus are lethal to the larvae of many lepidopterous insect pests. The potential use of these occluded viruses as entomopathogenic biocontrol agents has recently elicited a great deal of interest. The infectious virions of the nuclear-polyhedrosis viruses are protected by their random inclusion within an ordered protein matrix polyhedron. Knowledge of the composition of the polyhedral matrix protein(s) would facilitate the development of means to increase further the stability of the nuclear-polyhedrosis virus and thus prolong its virulence. Serological characterization would be useful in monitoring viral specificity to study its safety for use as an insecticide.

However, very little is known about the nature of these polyhedral matrix proteins, and conflicting reports on their properties have appeared (Bergold, 1963; Summers & Egawa, 1973; Padhi *et al.*, 1974; Kozlov *et al.*, 1975b). Some of the confusion about the number and molecular weights of the polyhedral matrix protein(s) can be traced to the variation caused by degradative enzymes in many of these virus protein preparations (Kuzin & Krzehova, 1948; Yamufugi *et al.*, 1957; Storer *et al.*, 1974; Eppstein & Thoma,

1975; Eppstein *et al.*, 1975; Summers & Smith, 1975; Kozlov *et al.*, 1975a).

Earlier we reported (Eppstein & Thoma, 1975) on the presence of a proteinase associated with the nuclear-polyhedrosis-virus preparations isolated from the cabbage looper, *Trichoplusia ni*. We described some of the properties of the proteinase (Eppstein & Thoma, 1975) and its degradation of the *T. ni* nuclear-polyhedrosis-virus matrix protein (Eppstein *et al.*, 1975). The properties of the proteinase and its subsequent degradation of the matrix protein appear to be similar to that reported by Summers & Smith (1975) for a *T. ni* granulosis virus. We have now further purified the intact matrix protein and have ascertained the initial site of degradation of the matrix protein by the endogenous proteinase. We also report on the greatly differing interactions of intact and cleaved matrix-protein subunits at low and high pH.

We have found two non-cross-reacting antigens on aggregates of the matrix protein. The largest aggregate at pH 9.5, which contains antigen I, appears to be held together by disulphide bond(s). Reduction of the aggregate causes it to dissociate, forming a smaller aggregate that contains an unrelated antigen II. The expression of the two unrelated antigens appears to reflect the different modes of aggregation of the protein subunit groups, rather than the existence of two distinct matrix proteins.

* To whom reprint requests should be sent. Current address: Department of Biological Sciences, University of California, Santa Barbara, CA 93106, U.S.A.

Materials and Methods

Preparation and isolation of T. ni nuclear-polyhedrosis-virus matrix protein

The single embedded *T. ni* nuclear-polyhedrosis-virus matrix protein was propagated and purified as previously described (Eppstein & Thoma, 1975), and three matrix-protein preparations were isolated from the nuclear-polyhedrosis virus as described by Eppstein *et al.* (1975). Preparation I was isolated in the absence of proteinase inhibition, preparation II was isolated with 5 mM-HgCl₂ as proteinase inhibitor, and preparation III was isolated with proteinase inhibition after the first 40 min of alkali dissolution of the nuclear-polyhedrosis-virus polyhedra.

Reduction and carboxymethylation

In one treatment, preparation I was reduced and carboxymethylated in 8.0 M-urea and 0.2% EDTA buffered at pH 8.5 as described by Hirs (1967). In another treatment, preparations I and III were reduced under non-denaturing conditions by dialysis against 0.01 M-Na₂CO₃/0.01 M-NaCl/0.01 M-dithiothreitol (pH 9.6) for 24 h at 4°C.

Polyacrylamide-gel electrophoresis

Analytical polyacrylamide-gel electrophoresis on 6 and 15% gels (5 mm × 50 mm) was conducted at room temperature (25°C) at pH 9.5 by the method of Davis (1964), except that the stacking gel was as a routine omitted. Immediately after electrophoresis, the gel was sliced into 2 mm slices, each slice was placed on top of a polymerized gel, and was immediately re-electrophoresed. Gels were stained for protein with Coomassie Brilliant Blue (Chrambach *et al.*, 1967) and for carbohydrate with the Schiff/periodate stain (Zacharius *et al.*, 1969).

Preparative electrophoresis was done at 4°C in 1.5 cm × 10 cm gels at 4 mA/gel for 8 h. Protein bands were sliced out after detection by their absorbance when irradiated with long-wavelength u.v. light, and the peptides were eluted from the pulverized gel slices by shaking at 4°C with electrophoresis gel buffer.

Electrophoresis was also conducted at pH 8.0, 8.5, 8.9 and 9.5 in a series of continuous pH buffer systems, with 0.375 M-Tris/HCl as the gel buffer and 0.0375 M-Tris/HCl as the reservoir buffer.

For a series of Ferguson plots (Chrambach & Rodbard, 1971) at pH 9.5, the appropriate gel concentrations (5–9%) were obtained by dilution of a stock 40% (w/v) acrylamide solution with a 1:30 (w/w) *NN'*-methylenebisacrylamide/acrylamide ratio (Hedrick & Smith, 1968).

Electrophoresis at pH 2.3 was performed by using photopolymerized 7.5% gels and Methyl Green as tracking dye (Parish & Marchalonis, 1970). The

dye position was marked before the gels were stained with Coomassie Blue.

SDS*/polyacrylamide-gel electrophoresis in 12% gels was performed with a discontinuous buffer system (Waehnelde & Mande, 1970), omitting the stacking gel and the K₄Fe(CN)₆ from the lower gel. The gels (6 mm × 90 mm) were left overnight under gel buffer before electrophoresis. Protein samples were dissolved in electrophoresis buffer containing 1.1% (w/v) SDS and 50 mM-dithiothreitol and were heated at 100°C for 3 min immediately before electrophoresis. Samples containing 5 mM-Hg²⁺ were reduced in 0.5 M-dithiothreitol. The position of the tracking dye after electrophoresis was marked, and the gels were stained with Coomassie Blue as described by Fairbanks *et al.* (1971) and destained in 7% (v/v) acetic acid.

The following proteins were run as standards: bovine serum albumin (mol.wt. 68000; Sigma, St. Louis, MO, U.S.A.); β -amylase (mol.wt. 50000; Worthington, Freehold, NJ, U.S.A.); carboxypeptidase A (mol.wt. 35000; Worthington); whale myoglobin (mol.wt. 17200; Sigma); cytochrome *c* (mol.wt. 12400; Sigma).

Isoelectric focusing

Isoelectric focusing was conducted in polyacrylamide gels as described by Wrigley (1971) by using LKB ampholytes nominally of pH range 5–8 (LKB, Pleasant Hill, CA, U.S.A.) and 7.5% gels with an *NN'*-methylenebisacrylamide/acrylamide ratio of 1:30 (w/w). Protein samples were incorporated into the gel matrix by photopolymerization.

Gel chromatography

Gel chromatography in 1.0 M-formic acid, pH 1.9, was performed at 4°C in a column (1.5 cm × 47 cm) packed with Bio-Gel P-30 or P-10 (100–200 mesh; Bio-Rad, Richmond, CA, U.S.A.). A 10 mg protein sample in 1 ml of column buffer was applied to the column and eluted at a flow rate of 7 ml/h. Fractions (1 ml) were collected and the elution profile was determined by the *A*₂₇₆ and *A*₄₅₀. Peaks that were to be rechromatographed were concentrated to approx. 1 ml by pervaporation (Kober, 1917) at 4°C. Gel chromatography was also performed in 1.0 M-formic acid by using calibrated (Andrews, 1964) Bio-Gel P-100 and P-10 (100–200 mesh) columns, and in 0.01 M-Na₂CO₃/0.01 M- or 0.05 M-NaCl (pH 9.5 or 9.6) on a calibrated column (1.5 cm × 87 cm) of Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.). Gel chromatography on Bio-Gel P-30 columns (1.5 cm × 46 cm) was also performed in 1.0 M-chloroacetic acid, pH 1.5, 1.0 M-sodium formate buffers, pH 2.4 and 2.6, 1.0 M-acetic

* Abbreviations: SDS, sodium dodecyl sulphate; dansyl (Dns), 5-dimethylaminonaphthalene-1-sulphonyl.

acid, pH2.8, and 1.0M-ammonium acetate buffers, pH3.0 and 3.3.

Chromatography in 6.0M-guanidine hydrochloride was performed by the method of Fish *et al.* (1969). A column (1.5cm×72cm) of Bio-Gel A-5m (100–200 mesh) was equilibrated at room temperature with 6M-guanidine hydrochloride (grade 1; Sigma)/0.1M-ammonium formate/1.0mM-dithiothreitol (Sigma), pH4.0. Elution volume was determined from the eluate weight and the measured solvent density. The column was calibrated by determining the elution volumes of carboxypeptidase A, myoglobin, lima-bean trypsin inhibitor [Sigma; average mol.wt. 9000 (Pusztai & Watt, 1970)] and insulin (Sigma; mol.wt. 5700).

Chromatography in phenol/acetic acid/water (1:1:1, w/v/v) was performed as described by Pusztai & Watt (1970), on a column (1.5cm×83cm) packed with Bio-Gel P-100 (100–200 mesh). The elution profile was determined by a turbidimetric protein assay (Fish *et al.*, 1969). The elution buffer was removed from the protein by successive 24h dialyses at room temperature against 1 litre each of 6.7M-, 1.7M- and 0.1M-acetic acid (Pusztai, 1966).

Chromatography in SDS was performed by the method of Fish *et al.* (1970). A column (1.5cm×102cm) packed with Bio-Gel A-1.5m or A-5m (100–200 mesh) was equilibrated with 0.05M-Tris/HCl/0.1% SDS/1.0mM-dithiothreitol, pH8.9. Samples were incubated in 0.1M-Tris/HCl/1.1% SDS/10mM-dithiothreitol, pH8.9, at 100°C for 3min before application to the column. The flow rate was 11ml/h, and 1ml fractions were collected. The column was calibrated with myoglobin, haemoglobin, cytochrome c, lima-bean trypsin inhibitor and insulin.

Preparation of fractions FI and FII

Matrix-protein preparation I (50mg in 5ml) was chromatographed on a column (2.5cm×87cm) packed with Bio-Gel P-30 equilibrated with 1.0M-formic acid at 4°C. The fractions of peak I and peak II were individually pooled, and are designated FI and FII respectively. Each of the pooled fractions was concentrated to approx. 1ml by pervaporation at 4°C, rechromatographed as described above and then freeze-dried from 1.0M-formic acid.

Absorption coefficient

The absorption coefficients $A_{1\text{cm}, 276\text{nm}}^{1\%}$ of fractions FI and FII were determined in 1.0M-formic acid, with bovine serum albumin as a standard, by using the protein assay of Lowry *et al.* (1951). To check for possible interference by a brown-coloured material (absorbing at 450nm) in determination of fraction FI, the absorption coefficient was determined on two samples that had different degrees of contamination. The A_{276}/A_{450} ratios of the two samples were 12 and 24. The absorption coefficient of fraction FII was also

determined on a dry-weight basis after drying 4.05mg of the protein at 110°C for 24h.

N-Terminal analysis

The intact matrix-protein subunit was isolated by SDS/polyacrylamide-gel electrophoresis of preparation II, which had been isolated with proteinase inhibition. N-Terminal analysis was performed by the method of Weiner *et al.* (1972), except that polyamide-6 sheets (20cm×20cm) were used (Brinkman Instruments, Westbury, NY, U.S.A.). The dansylated proteins were hydrolysed in 6.7M-HCl at 100°C for 4h and for 16h. Since the R_F of the dansyl-amino acids increased markedly with loading concentration, especially in benzene/acetic acid (9:1, v/v), the identities of all unknown spots were confirmed by co-chromatographing with known standards. Dansyl-amino acid standards were obtained from Sigma and Nutritional Biochemicals (Cleveland, OH, U.S.A.).

Analytical ultracentrifugation

Sedimentation-velocity experiments were run at 20°C in 1.0M-formic acid or in 0.01M- Na_2CO_3 /0.01M- or 0.05M-NaCl at pH9.5 or 9.8, in the Spinco model E analytical ultracentrifuge equipped with electronic speed control, at 56000rev./min in the An-D rotor, with a Kel-F single-sector centrepiece and quartz windows. Photographs were taken with u.v.-absorption optics aligned at the midpoint focus of the camera lens, on Kodak commercial film 6127. The negatives were traced with a Beckman model RB Analotrol with the film densitometer attachment. The sedimentation coefficients were calculated by standard procedures (Chervenka, 1969).

Molecular-weight determinations were performed by the meniscus-depletion sedimentation-equilibrium centrifugation technique of Yphantis (Yphantis, 1964; Chervenka, 1969). The FII sample (0.6–1.2mg/ml) was dissolved in 1.0M-formic acid containing 0.1% sucrose to provide a stabilizing gradient (Szuchet & Yphantis, 1973). The experiments were run at 20°C and 52000rev./min, in the An-D rotor with a 12mm aluminium-filled Epon double-sector centrepiece with sapphire windows. A 6mm column height was used. Baseline determinations were made at the termination of each run. Before disassembly, the cell was shaken for 5min, centrifuged at 3200rev./min for 20min, and then accelerated to 52000rev./min for the baseline photograph to correct for window distortion (Chervenka, 1969; Teller, 1973). Kodak spectroscopic plates (type II-G) were used; exposure times of 5min were necessary. The fringe displacements were measured after 24, 42 and 72h by using a model GC Nikon profile projector comparator. Three measurements were made for each of the 40–50 radial distance measurements on both the sample and the baseline plates. The

molecular weight was determined at each measured point along the concentration gradient in the cell for each five successive radial distance measurements, and was then extrapolated to the molecular weight at infinite dilution.

The partial specific volume was calculated from the amino acid composition (Schachman, 1957), and the solvent density was calculated from data in the literature (Chervenka, 1969; Chemical Rubber Co., 1974).

Amino acid analysis

Samples (1–3 mg in 1 ml) were hydrolysed *in vacuo* in 6M-HCl at 110°C for 24, 48 and 72 h. Samples in polyacrylamide gels were sliced out after staining and were prepared for amino acid analysis by the method of Spiro (1973). Corresponding bands from six or more gels were combined. The amino acid analysis was done with Durram-IA resin by the single-column methodology (Durram, Palo Alto, CA, U.S.A.) by using the modified ninhydrin assay of Rosen *et al.* (1962), and measuring the A_{405} (Ellis & Garcia, 1971). The large ammonia peak and other ninhydrin-positive by-products from the hydrolysed polyacrylamide gels prevented determination of the basic amino acids in samples hydrolysed in polyacrylamide-gel sections.

Tryptophan was determined spectrophotometrically (Edelhoc, 1967).

Carbohydrate, DNA and phosphate analysis

Total carbohydrate was analysed by the phenol/ H_2SO_4 assay (Dubois *et al.*, 1956). Neutral sugars were determined in fraction FI by descending paper chromatography (Spiro, 1966); amino sugars were determined both by descending paper chromatography (Mukerjee & Sri Ran, 1969) and by the Morgan-Elson and Elson-Morgan reactions (Davidson, 1966; Good & Bessman, 1964). Sialic acid was assayed by the thiobarbiturate assay (Warren, 1954). DNA analysis was performed by the method of Schneider (1957), and total phosphate was assayed by the method of Ames (1966).

Serological studies

Antiserum to preparation-I matrix protein was produced in rabbits as described by Scott & Young (1973). Immunoelectrophoresis and immunodiffusion were performed by the micro-slide technique of Scheidegger (Gelman Instrument Co., 1968; Ouchterlony, 1968). The equivalent antigen-antibody combining ratios were determined by double-diffusion techniques (Ouchterlony, 1968; Williams & Chase, 1971). The antigen and antibody were allowed to diffuse for 36 h at room temperature before staining with Amido Black.

In addition to Ouchterlony double-diffusion and conventional immunoelectrophoresis, preparation-I matrix-protein samples were subjected to polyacryl-

amide-gel electrophoresis on 6% gels at pH 9.5, as described above, followed by double immunodiffusion.

Results

Cleavage of monomer subunit: low-pH dissociation

Purification of monomer subunit. Preparation-II matrix protein (isolated with proteinase inhibition) was shown previously by SDS/polyacrylamide-gel electrophoresis to contain one prominent peptide of 28000 ± 3000 daltons, a lesser peptide of 55000 ± 4000 daltons and several minor peptides smaller than 19000 daltons (Eppstein *et al.*, 1975). We have now further purified this preparation by chromatography on a Bio-Gel P-10 column in 1.0M-formic acid. The material excluded from the column (95% of the total sample) contained only the 28000- and 55000-dalton peptides. We believe that this 28000-dalton peptide is the undegraded monomeric subunit of the matrix protein, and that the 55000-dalton peptide is possibly a dimer, since the peptide bands have similar amino acid compositions, within the limits of error to be expected of analyses of material eluted from gels (Table 1).

Gel chromatography. Chromatography of preparation I (isolated with no proteinase inhibition) on Bio-Gel P-30 in 1.0M-formic acid, pH 1.9, separated it into two peptide fractions, FI and FII (Eppstein *et al.*, 1975). Fraction FI contained several peptides but fraction FII contained a single peptide (Eppstein *et al.*, 1975), yet both fractions FI and FII gave single peaks on rechromatography. The apparent mol.wt. of fraction FI was 65000, on the basis of its elution volume at pH 1.9 from a calibrated Bio-Gel P-100 column. Fraction FII had mol.wt. 9400 when measured by sedimentation-equilibrium centrifugation at pH 1.9 (see below).

Both the elution volume and the relative amount of free fraction FII released from preparation I were dependent on pH (Table 2). At pH 2.6 or less, the elution volume (V_e) divided by the total column volume (V_t) was 0.8, but this fell to 0.6 at pH 2.8 and above. Samples of fraction FII isolated at pH 3.0 and 3.3 ($V_e/V_t = 0.6$) gave a single peak on rechromatography at pH 1.9, with $V_e/V_t = 0.8$. Lowering the pH below 1.9 did not change either the elution volume of fraction FII or the amount released from preparation I, so we assume that the sample was completely fractionated at this pH.

Although fraction FII appeared to be completely separated from fraction FI by gel chromatography, it must have been equilibrating with the fraction FI aggregate during chromatography. This pH-dependent interaction caused the elution volume of fraction FII to change (Halvorson & Ackers, 1974; Zimmerman, 1974) between pH 2.6 and 2.8. Also, fraction FII was essentially completely dissociated from fraction FI below pH 2.6 (Table 2). Therefore

Table 1. *Amino acid composition of matrix-protein subunit and aggregates*

The amino acid compositions (for neutral and acidic amino acids only) are given in mol of amino acid/mol of polypeptide equivalent (28 300g).

Amino acid	Subunits from SDS/polyacrylamide-gel electrophoresis			Aggregates from electrophoresis at pH9.5		
	28 000-mol.wt. peptide (prepn. II)	31 000 (±3000)-mol.wt. peptide* (prepn. I, Fig. 2b)	56 000-mol.wt. peptide* (prepn. II)	B1	BS	B3*
Aspartic acid	27.0	28.2	26.6	31.1	28.6	27.9
Threonine	7.2	7.9	10.0	7.2	8.2	9.7
Serine	13.1	11.7	16.7	16.0	11.9	14.6
Glutamic acid	24.0	24.0	23.9	21.8	22.1	23.1
Proline	15.2	14.7	14.2	18.4	16.4	—
Glycine	—	—	—	—	—	—
Alanine	12.6	12.3	13.3	—	13.5†	—
Half-cystine	—	—	—	—	—	—
Valine	11.6	12.8	15.7	13.3	11.2	13.8
Methionine‡	2.9	—	2.5	—	3.6	—
Isoleucine	7.7	8.2	9.3	7.2	7.3	8.3
Leucine	16.8	16.6	16.3	18.2	16.7	16.9
Tyrosine	11.6	—	8.9	13.1	13.5	11.4
Phenylalanine	11.3	12.0	9.5	11.0	12.1	11.9

* A small amount of sample was available for analysis; therefore the results are not as accurate as from other analyses.
† Value for alanine may be high owing to large adjacent glycine peak because of large gel sample (and therefore glycine buffer) hydrolysed. See the text for experimental details.
‡ Values for methionine may be low owing to oxidative losses.

Table 2. *pH-dependence of elution volume of fraction FII and concentration of free FII peptide*

V_e/V_t = elution volume/total column volume of Bio-Gel P-30 column. The amount of fraction FII is expressed as mg dissociated from 10mg of preparation-I matrix protein, determined from the A_{276} and the extinction coefficient of fraction FII.

pH	V_e/V_t	Amount of FII peptide (mg)
1.5	0.80	3.0
1.9	0.80	3.3
2.4	0.78	3.2
2.6	0.77	3.2
2.8	0.58	3.2
3.0	0.60	2.5
3.3	0.57	2.2

we infer that there is no appreciable interaction between fractions FI and FII below pH 2.6. We found progressively smaller amounts of free fraction FII above pH 2.8, reflecting the pH-dependent association between fractions FI and FII.

Chromatography of preparation I on Bio-Gel A-5m in 6.0M-guanidine hydrochloride/1.0mM-dithiothreitol at pH 4.0 and on Bio-Gel P-100 in phenol/acetic acid/water (1:1:1, w/v/v) also resolved fractions FI and FII. The mol.wt. of the FII peptide was approx. 9000, on the basis of its elution volume from the calibrated guanidine hydrochloride column.

Fig. 1. *Preparation-II matrix protein (isolated with proteinase inhibition) chromatographed on Bio-Gel P-100 at pH1.9*

Preparation II (approx. 7 mg in 1 ml of column buffer) was first chromatographed on a Bio-Gel P-10 column (1.5cm×47cm) in 1.0M-formic acid; the single peak eluted with the void volume was concentrated by pervaporation, and was rechromatographed on a Bio-Gel P-100 column (1.5cm×46cm) in 1.0M-formic acid, pH1.9, at room temperature (latter chromatogram shown).

Chromatography of preparation II (isolated with proteinase inhibition) on Bio-Gel P-10 and P-100 columns in 1.0M-formic acid gave a single broad peak that was eluted in the void volume (Fig. 1). Thus the apparent aggregate mol.wt. at pH1.9 of

the non-degraded preparation II was greater than 100000, which indicates that the aggregate contains at least four monomer units. The tailing of this peak suggests an equilibrium between the aggregate and its subunits, with the aggregate in greatest concentration (Zimmerman, 1974; Zimmerman & Ackers, 1971).

Absorption coefficient and yields of fractions FI and FII. When measured by the Lowry *et al.* (1951) protein assay, $A_{1\text{cm}, 276\text{nm}}^{1\%} = 15.6 \pm 0.4$ for fraction FI and $A_{1\text{cm}, 276\text{nm}}^{1\%} = 11.2 \pm 0.4$ for fraction FII. The absorption coefficient for fraction FII determined on a dry-weight basis was $A_{1\text{cm}, 276\text{nm}}^{1\%} = 11.1 \pm 0.2$.

From these absorption coefficients, the relative weights of the peptides in fractions FI and FII were calculated by summing their total respective absorbance at 276nm from the chromatogram in which fractions FI and FII were completely separated. The ratio of the total summed absorbance at 276nm, $A_{\text{FI}}/A_{\text{FII}}$, was 2.76 ± 0.01 , and dividing this ratio by the ratio of the above absorbance

coefficients gives the weight ratio 2g of fraction FI/g of fraction FII.

Peptide composition of fractions FI and FII. SDS/polyacrylamide-gel electrophoresis revealed that fraction FI was an aggregate of three major and several minor peptides (Eppstein *et al.*, 1975). The most prominent peptide had mol.wt. 16000 ± 2700 (Fig. 2b). Chromatography of fraction FI on Bio-Gel A-5m or A-1.5m in SDS resulted in the isolation of a fraction that contained only the 16000 ± 2700 -dalton peptide when analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 2d). The largest peptide of 31000 ± 3000 daltons has the same amino acid composition and mol.wt. (± 3000) as the intact 28000-dalton subunit (Table 1), and probably represents monomer which had not yet been cleaved by the proteinase.

Fraction FII contained a single peptide, as indicated by SDS/polyacrylamide-gel electrophoresis (Fig. 2c) and confirmed by electrophoresis at pH 2.3 (Fig. 2e). Fraction FII had the same amino acid composition as the peptide of corresponding mobility obtained from SDS/polyacrylamide-gel electrophoresis of preparations I and III (Eppstein, 1975).

N-Terminal analysis. Both the 28000-dalton subunit, isolated as one peptide band from SDS/polyacrylamide-gel electrophoresis of preparation II, and fraction FII, isolated by chromatography of preparation I, contained only one *N*-terminal amino acid, tyrosine; Fraction FII may be the fragment derived by proteolytic cleavage from the *N*-terminus of the intact subunit (Eppstein *et al.*, 1975).

The fraction FI aggregate, which separated into three main peptides and several minor peptides (Fig. 2b) on SDS/polyacrylamide-gel electrophoresis, contained six different *N*-termini. The three predominant *N*-terminal amino acids were phenylalanine, tyrosine and leucine. The *N*-terminal phenylalanine was present in greatest quantities, as judged by the brightness of the Dns-phenylalanine spot, and probably came from the 16000-dalton peptide, which was the main component of fraction FI. The *N*-terminal tyrosine probably came from the undegraded monomer present. Dns-methionine, Dns-alanine and $\alpha\epsilon$ -bis-Dns-lysine were present at the lower limits of detectability, approximately 100-fold less (determined by dilution) than the concentrations of Dns-tyrosine and Dns-phenylalanine, and probably represent three of the minor peptides present in the fraction FI aggregate.

Sedimentation-coefficient determination. The sedimentation coefficient of the fraction FI aggregate in 1.0M-formic acid was $s_{20, \text{buffer}} = 5.3 \pm 0.1\text{S}$. Fraction FII, a single component, sedimented at pH 1.9 with $s_{20, \text{buffer}} = 0.58 \pm 0.04\text{S}$.

Molecular-weight determination. A technique commonly used for molecular-weight determinations of subunits is polyacrylamide-gel electrophoresis in

Fig. 2. Polyacrylamide-gel electrophoresis of *T. ni* matrix protein

(a)–(d) SDS/polyacrylamide-gel electrophoresis of 0.2–0.5 mg samples on 12% gels. Electrophoresis was towards the anode. (a) Preparation-I matrix protein; (b) fraction FI; (c) fraction FII; (d) 16000-dalton peak from SDS chromatography of fraction FI on Bio-Gel A-1.5m; (e) pH 2.3 electrophoresis (no SDS) of fraction FII. Electrophoresis was towards the cathode for (e).

the presence of SDS (Weber *et al.*, 1972). However, fluctuations in the stoichiometry of SDS/protein ratios (Tung & Knight, 1972; Weber *et al.*, 1972) can lead to errors in molecular-weight determinations. Hence molecular weights determined in the presence of SDS should be confirmed by a different method, preferentially ultracentrifugal analysis.

Since complete dissociation of the intact matrix protein was only accomplished in SDS, a subunit molecular weight would have to be determined in the presence of SDS. Because SDS binds tightly to proteins (Weber & Kuter, 1971), the partial specific volume of the SDS-protein complex and the ratio of SDS to protein in the complex must be determined (Steers *et al.*, 1965) before the molecular weight by sedimentation-equilibrium centrifugation can be determined. Experimental difficulty of accurately measuring these values has been reported to lead to large errors in the factor $(1 - \bar{v}\rho)$ (where $\bar{v}$ is partial specific volume and ρ is density), with corresponding errors in molecular-weight values. Consequently, we determined the matrix-protein subunit molecular weight by measuring the molecular weight of a purified subunit fragment (FII) comprising one-third of the matrix protein. Since fraction FII was readily dissociated in low-pH buffers, SDS was not necessary to prevent aggregation.

With a 6mm column height, sedimentation equilibrium was obtained with fraction FII after 24h centrifugation at 52000 rev./min. The partial specific volume of fraction FII was calculated to be 0.734 ml/g from the amino acid composition (Schachman, 1957), the density of the 1.0M-formic acid/0.1% sucrose solvent was calculated to 1.0067 g/ml (Chervenka, 1969; Chemical Rubber Co., 1974) and the weight-average molecular weight at infinite dilution of fraction FII was 9430 ± 120 . A typical plot of $\log(\text{fringe displacement})$ versus $(\text{radial distance})^2$ is shown in Fig. 3. The sample appeared to be homogeneous, non-self-associating and only slightly non-ideal, as indicated by the very slight concave-upward curvature of the plot (Chervenka, 1969). The apparent molecular weight of the fraction FII peptide remained constant for 24h, 42h and 72h sedimentation periods.

The molecular weight of fraction FI could not be determined in the ultracentrifuge because of precipitation of some of the sample during centrifugation. However, since it was shown above that the fraction FII peptide of 9430 ± 120 daltons constituted one-third of the total matrix-protein weight, the mol.wt. of the intact matrix-protein subunit is then $3 \times (9430 \pm 120)$ or 28300 ± 400 . This value is in good agreement with the mol.wt. of 28000 ± 3000 obtained from SDS/polyacrylamide-gel electrophoresis.

Carbohydrate, DNA and phosphate analysis. A small amount of carbohydrate was associated with the

Fig. 3. Meniscus-depletion sedimentation-equilibrium centrifugation of fraction FII

Freeze-dried fraction FII was dissolved (0.7mg/ml) in 1.0M-formic acid/0.1% sucrose, and was centrifuged at 19.8°C for 24h at 52000 rev./min in a Spinco model E analytical ultracentrifuge. Equilibrium photographs were taken after 24h by using Rayleigh interference optics. The data were plotted, after baseline correction, as $[\log(\text{fringe displacement}) + 3]$ versus $(\text{radial distance})^2$. Three points were plotted for each radial distance. The molecular weight at infinite dilution was 9430 ± 120 .

matrix-protein preparation I. Fraction FI contained the neutral sugars mannose, glucose and galactose in the approximate molar proportions 2:1:1, and the amino sugar galactosamine. No sialic acid was present. The carbohydrate content of the matrix protein after isolation at pH 9.5 was approx. 3% by wt. Fraction FI obtained by chromatography at pH 1.9 initially contained 1% carbohydrate, but after rechromatography contained only 0.5% carbohydrate. A decrease in carbohydrate content was concomitant with a decrease in a tightly absorbed brown-coloured material, which suggests that the carbohydrate is associated with the chromogen. Release of the chromogenic substance was measured by an increase in A_{276}/A_{450} of the protein. Fraction FII contained no carbohydrate and no material that absorbed at 450 nm.

No DNA was found in matrix-protein preparation

I. A very small amount of phosphate was detected in preparation I, approx. 1 mol of phosphate/30000 g of unfractionated preparation I.

Evidence for aggregate structure at pH 9.5

Polyacrylamide-gel electrophoresis. Preparation I (isolated with no proteinase inhibition) on electrophoresis on 6% gels (pH 9.5) gave one predominant band (Fig. 4, B2), two minor bands (B1 and B3), two trace bands (B4 and B5) and some bands that migrated with the tracking dye. Bands B1–B4 stained with the Schiff/periodate reagents and are probably glycoproteins. When material from bands B1, B2 and B3 was re-electrophoresed immediately (as gel slices; electrophoresis time 45 min), it migrated as single bands.

However, when material from bands B1–B4 was eluted and re-electrophoresed approx. 24 h after isolation, bands B1–B3 each gave four bands that migrated with R_m values characteristic of bands B1, B2, B3 and the dye. When band-B4 material was re-electrophoresed, bands B2 and B4 were obtained. After 5 weeks at 4°C, electrophoresis of band-B2 material yielded a banding pattern indistinguishable from that obtained with unfractionated preparation I. Since material from all of the bands interconverted, we propose that bands B1–B4 represent aggregates of a common set of peptides. The time course of band interconversions suggests that the aggregates and peptides slowly equilibrate. Significant interconversion of the peptide bands had occurred after 24 h at 4°C, but longer periods were necessary to obtain what appears to be the equilibrium distribution.

Traces of proteinase that migrate with some of the bands (Eppstein & Thoma, 1975) cannot account for

the interconversion of the bands; proteolytic degradation would cause some bands to appear at the expense of others, but not interconversions among bands. The multiple banding pattern obtained with preparation I was not an artifact of the electrophoresis system, since the same general pattern was obtained by using photopolymerized gels and continuous pH-buffer systems from pH 8.0 to 9.5.

Reduction of matrix-protein preparation I under non-denaturing conditions resulted in an increase in band B4 and decrease in band B2, but band B2 was still dominant. Reduction under denaturing conditions (urea) resulted in complete disappearance of band B2 with a concomitant increase in band B4. Reduced (under non-denaturing conditions) preparation III also contained bands B2 and B4, but band B4 was dominant. The greater proportion of band B4 in preparation III may have been related to residual Hg^{2+} bound to the peptides in that preparation, which may have interfered with more extensive aggregation.

The Fergusson plots (Chrambach & Rodbard, 1971) for bands B2–B5 yielded lines which statistically had a common intercept at 3.6% gel concentration (Fig. 5a). A correlation-regression analysis (program CORREG; University of Arkansas Computing Statistics Manual) showed that there was no statistical difference in the common intercept (3 and 116 degrees of freedom). The normal interpretation of these results is that bands B2–B5 are size isomers which are not retarded in a 3.6% gel (Hedrick & Smith, 1968). The Fergusson plots for bands B1 and B2 were parallel, implying that the aggregates of these bands are of the same molecular size but have different charges.

Fig. 4. Polyacrylamide-gel electrophoresis of preparation-I matrix protein
 ----, Densitometer trace of electrophoresis of 100 μ g of preparation I on 6% gel with a 1:30 (w/w) *NN'*-methylenebisacrylamide/acrylamide ratio. Bands B1–B5 are shown. —, Scan of a gel overloaded with respect to band B2 in order to show bands B4 and B5 more clearly.

Fig. 5. Ferguson plots for matrix protein at pH 9.5

Preparation-I matrix protein (100 μ g) was electrophoresed at pH 9.5 on 5–9% polyacrylamide gels. In (a) the results were plotted by using a least-squares fit to a plot of $\ln(1000R_m)$ versus percentage gel concentration for each peptide band. Each point shown represents the average of three to twelve R_m determinations. The lines were analysed for a common intercept at 3.6% gel concentration by using a correlation-regression analysis from which the restricted error mean square was compared with the sum of the non-restricted (least-squares) error mean squares (program CORREG, University of Arkansas, Computing Statistics Manual). For 3 and 116 degrees of freedom, an F value of 0.292 was obtained, which is never statistically significant. Thus no statistical difference was found in the 3.6% common intercept for bands B2–B5. The inset shows a 6% gel, showing the bands used in the plots. $\square$, B1; $\triangle$, B2; $\circ$, B3; $\blacksquare$, B4; $\blacktriangle$, B5. (b) The slopes obtained for lines B2–B5 were plotted against eight, six, four and two monomer-unit equivalents respectively. This ratio of monomer units gave the best fit, with a correlation coefficient of 0.9998.

The slope of a plot of $\log R_m$ versus gel concentration is proportional to the mol.wt. of a protein (Hedrick & Smith, 1968). The Ferguson plots were consistent with the simplest monomer-combining ratio of 4:3:2:1 for bands B2–B5 respectively. The mol.wt. of the monomer was previously shown to be 28000, and band B4 has a mol.wt. of approx. 100000 (see below). Therefore band B4 is probably composed of four monomer-unit equivalents. It follows that bands B2–B5 contain eight, six, four and two monomer-unit equivalents respectively (Fig. 5b).

Isoelectric focusing

The preparation-I matrix protein was mainly eluted in the void volume of a Sephadex G-100 or Bio-Gel P-200 column at pH 9.5. The aggregates showed only one species on isoelectric focusing. The pI was approx. 5.8, which is similar to that obtained with other insect nuclear-polyhedrosis virus-matrix proteins (Smith, 1967). Thus the degraded peptides apparently associate to form one or more species which have a common isoelectric point. This confirms our postulate that the bands found on polyacrylamide-gel electrophoresis are size isomers.

Amino acid analysis. The amino acid compositions of aggregates B1, B2 and B3 (Fig. 4) were very similar to or identical with each other and that of the

intact 28000-dalton subunit (Table 1). The preparation I aggregates were different in amino acid composition from the peptide fragments (FI and FII) dissociated from preparation I at pH 1.9 (Eppstein *et al.*, 1975). These results are consistent with our hypothesis that proteolytic cleavage does not cause extensive disaggregation of the matrix protein at pH 9.5.

Analytical ultracentrifugation. Preparation-I matrix protein eluted in the void volume on a Bio-Gel P-200 column sedimented at $11.0 \pm 0.1S$ at pH 9.5. Band B2, which was isolated after preparative electrophoresis at pH 9.5 of preparation I, sedimented with $s_{20, \text{buffer}}$ of $10.9 \pm 0.1S$.

When the pH was raised slightly, preparation-I matrix protein became highly dissociated. At pH 9.8, its main component sedimented with $s_{20, \text{buffer}} = 1.4S$.

Serological evidence for two antigens

Antigens associated with gel-chromatography peaks. Preparation-I matrix protein, eluted in the void volume on a Sephadex G-100 column (pH 9.5), contained a major antigen I and a minor antigen II (Plate 1a). However, the fractions eluted in the tailing end (100000 daltons) of the void-volume peak contained only the minor antigen II (Plate 1b). Thus the aggregate containing antigen II (band B4, see below) had a mol.wt. of approx. 100000. The aggregate

eluted in the void volume from the Bio-Gel P-200 column (≥ 200000 daltons) was almost completely separated from the smaller aggregate that contained antigen II (Plate 1c). The precipitin arcs for antigen I and antigen II crossed, indicating that the two antigens shared no common determinants (Ouchterlony, 1968; Williams & Chase, 1971).

The two peptide fragments (FI and FII) dissociated from preparation I at pH 1.9 did not show any antigenic activity at pH 8.8.

Antigens associated with bands B1–B5 isolated from preparation I by polyacrylamide-gel electrophoresis. Electrophoresis of preparation-I matrix protein on polyacrylamide gels (pH 9.5), followed by immunodiffusion, permitted the identification of the peptide bands that contained antigen I and antigen II (Plate 1d). Antigen I was primarily associated with band B2, but smaller amounts were associated with bands B3 and B5. Antigen II was primarily localized on band B4.

To confirm that antigen I was localized on band B2 and that antigen II was localized on band B4, bands B1–B5 were immediately re-electrophoresed (as gel slices) and then subjected to immunodiffusion. Each freshly isolated band showed the presence of one antigen. However, 24 h after elution from a preparative gel, immunodiffusion and immunoelectrophoresis of band B2 showed the presence of a small amount of antigen II in addition to antigen I (Plate 1e), whereas band B4 only contained antigen II (Plate 1f). The centres of the two arcs obtained from band B2 on immunoelectrophoresis had different R_m values, indicating that the species containing antigen II had dissociated and partially separated from the band-B2 aggregate that contained antigen I.

Antigens obtained from preparation III. Reduced preparation-III matrix protein gave the same two precipitin arcs as were obtained with preparation I, except that antigen II was more prominent than antigen I (Plate 1g). The predominance of antigen II in reduced preparation III is accordingly consistent with its electrophoretic banding pattern. Band B4 (containing antigen II) was the major band present, and band B2 (containing antigen I) was present at much smaller concentrations.

Discussion

Limited proteolysis of monomer subunit

SDS/polyacrylamide-gel electrophoresis of the matrix-protein preparations revealed the effects and specificity of the endogenous proteinase. Preparation II showed very little degradation of the 28000-dalton monomer, preparation III showed limited degradation of the monomer into two main peptides of 16000 ± 2600 and 12000 ± 2600 daltons, whereas preparation I was almost completely degraded by the

proteinase into peptides of less than 28000 daltons (Eppstein *et al.*, 1975).

It appears that a single bond in the matrix protein is most susceptible to cleavage by the proteinase. When the polyhedra were initially added to the Na_2CO_3 , the pH was 11.0, where the proteinase possessed 30% of maximal activity; after 40 min most of the protein had dissolved, and the pH had fallen to 10.7, where the proteinase possessed 65% of maximal activity (Eppstein & Thoma, 1975). Apparently during the first 40 min of dissolution of the polyhedra in Na_2CO_3 , the proteinase had degraded a significant fraction of the intact matrix protein into two main fragments of 18000 and 9000 daltons. The larger of the two main fragments (Fig. 2d) was then slowly hydrolysed by the proteinase to give the smaller fragments apparent in fraction FI (Fig. 2b). The smaller of the two main fragments (Fig. 2c) is identical with the FII peptide of 9400 daltons. The total amount of fraction FII obtained from preparation I at pH 1.9 remained constant, indicating that fraction FII was not further cleaved by the proteinase.

If the proteolytic degradation of the matrix protein yields peptide FII from the *N*-terminus and a peptide aggregate FI which represents the rest of the protein, then the sum of the molar amino acid compositions of fractions FI and FII should be the same as the composition of the intact matrix-protein subunits. As we reported (Eppstein *et al.*, 1975), this was the case.

Interactions of intact and cleaved monomer subunits.

Preparation-I matrix protein, isolated without proteinase inhibition, contained very little of the intact matrix-protein subunit. Cleavage by the endogenous proteinase resulted in the formation of a peptide mixture which was highly self-associating. The experimental evidence indicates that the proteolytic peptide fragments and the intact subunits were held together by both hydrophobic and ionic interactions.

We found that in preparation I a non-covalent interaction between the FII peptide and the FI aggregate was disrupted in 1.0M-formic acid. The association was sensitive to a group(s) ionizing above pH 2.6 (Table 2). The FII peptide could also be dissociated from fraction FI by 6.0M-guanidine hydrochloride at pH 4, by phenol/acetic acid/water, as well as by SDS. However, dissociation of the FI aggregate into separate peptides (Figs. 2b and 2d) and dissociation of the intact matrix protein into its monomeric subunits was only complete in SDS. This suggests that strong hydrophobic forces contribute to the association of the degraded FI peptides in preparation I, and in causing the association of the intact subunits in preparation II.

EXPLANATION OF PLATE I

Immunoelectrophoresis and immunodiffusion of matrix-protein preparations I and III

The origin (cathode end) of the immunoelectrophoreses is indicated by the right edge of the slide. (a) Sephadex G-100 void-volume peak (preparation I); (b) tailing end (100000-dalton region) of Sephadex G-100 void-volume peak; (c) Bio-Gel P-200 void-volume peak (preparation I); (d) polyacrylamide-gel electrophoresis of preparation I, followed by immunodiffusion. Location of electrophoresis bands B2 and B4, and top of gel (—) and dye band (+) are shown. (e) Immunoelectrophoresis of bands B2 24 h after elution from polyacrylamide gel; (f) immunodiffusion of band B4 after elution from polyacrylamide gel; (g) immunoelectrophoresis of preparation III.

Aggregation of matrix protein

At pH 9.5, the proteinase-degraded and the intact matrix protein have similar electrophoretic properties. Both preparations exist as a mixture of polymers. The isolated aggregates from the proteinase-degraded preparation-I matrix protein had essentially the same amino acid composition as the undegraded matrix-protein subunit (Table 1). Since very little or none of the intact subunit remained in preparation I (Eppstein *et al.*, 1975), it can be concluded that at pH 9.5 the peptides of the proteolytically cleaved monomer units remain associated in monomer equivalents. Since these degraded units aggregate in monomer equivalent units, they probably maintain the basic structure of the unhydrolysed subunit at pH 9.5.

Antigens present in aggregates

The self-association of the peptides of the hydrolysed subunits was confirmed serologically. The dissociated fragments (FI and FII) from preparation-I matrix protein showed no antigenic reactivity, whereas the aggregates of these degraded peptides contained two non-cross-reacting antigens (Plate 1).

The presence of two serologically unrelated antigens does not necessarily indicate the presence of two different proteins; the antigenic specificity of a protein may change according to the state of aggregation of the protein (Munoz, 1959; Krywienczyk & Bergold, 1961; Goodman, 1967; Rapport & Zaitlin, 1970; Benjamini *et al.*, 1972; Summers & Egawa, 1973). The production of antibodies is sometimes sensitive to the quaternary structure of antigens. With tobacco-mosaic virus (Rapport & Zaitlin, 1970; Benjamini *et al.*, 1972), distinct antigenic states could be correlated with the degree of polymerization of the identical tobacco-mosaic-virus protein subunits.

We have shown that band B2 was partially depolymerized by reduction of disulphide bonds to give band B4. The potential expressions of both antigens I and II were contained on peptides present in band B2. Antigen I was expressed when the peptides were aggregated to form band B2. Dissociation of a smaller aggregate, B4, from band B2 gave rise to the immunological expression of antigen II. The dissociation of band B2 to form band B4 was greatly enhanced by reduction. We infer that either (1) intermolecular disulphide bonds join B4 aggregates to form the B2 aggregate, or (2) the formation of intramolecular disulphide bonds causes a conformational change resulting in aggregation to form band B2.

Forces contributing to aggregate formation

The non-covalent association of the cleaved peptides of preparation-I matrix protein may be

reinforced by covalent disulphide-bond formation. However, the association of the degraded peptides into subunit equivalents cannot be entirely dependent on disulphide-bond formation, since a purified peptide (FII), which comprises one-third of the total weight of the degraded preparation-I matrix protein, does not contain any cysteine (Eppstein *et al.*, 1975).

We have shown that association of the degraded matrix-protein subunits is chiefly due to hydrophobic interactions, with a small contribution by ionic interactions (groups with pK approx. 2.5–3.5). The dissociation of the degraded peptides of preparation I at pH 9.8 but not 9.5 suggests that another set of ionic groups, with pK near pH 9.8, is also important in self-association. Thus both ionic and hydrophobic interactions as well as disulphide bonds contribute to the association of the matrix-protein aggregates.

We thank Howard A. Scott and Seth Y. Young, III, at the Virology and Biocontrol Laboratory of the University of Arkansas for the preparation and isolation of the nuclear-polyhedrosis-virus polyhedra and the preparation-I matrix protein, and Howard A. Scott for performing the immunoelectrophoreses. We thank Jimmy D. Allen for writing the computer programs, for drawing Figures, and also for many helpful discussions with D. A. E. We thank Francis S. Millett and Nicole Staudenmayer for their help with the amino acid analyses, and the University of Arkansas Computing Center for computer facilities. This work was partially supported by a National Defense Education Act Title IV Research Fellowship to D. A. E.

References

- Ames, B. N. (1966) *Methods Enzymol.* **8**, 115–118
- Andrews, P. (1964) *Biochem. J.* **91**, 222–233
- Benjamini, E., Sciblerski, R. J. & Thompson, K. (1972) *Contemp. Top. Immunochem.* **1**, 1–49
- Bergold, G. H. (1963) in *Insect Pathology, an Advanced Treatise* (Steinhaus, E. A., ed.), vol. 1, pp. 413–456, Academic Press, New York
- Chemical Rubber Co. (1974) *Handbook of Chemistry and Physics*, 54th edn., Cleveland, OH
- Chervenka, C. H. (1969) *A Manual of Methods for the Analytical Ultracentrifuge*, Spinco Division of Beckman Instruments, Palo Alto, CA
- Chrumbach, A. & Rodbard, D. (1971) *Science* **172**, 440–451
- Chrumbach, A., Reisfeld, R. A., Wyckoff, M. & Zaccari, J. (1967) *Anal. Biochem.* **20**, 150–154
- Davidson, E. A. (1966) *Methods Enzymol.* **8**, 52–60
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. & Smith, F. (1956) *Anal. Chem.* **28**, 350–356
- Edelhoc, H. (1967) *Biochemistry* **6**, 1948–1954
- Ellis, J. P. & Garcia, J. B., Jr. (1971) *J. Chromatogr.* **59**, 321–327
- Eppstein, D. A. (1975) Ph.D. Thesis, University of Arkansas
- Eppstein, D. A. & Thoma, J. A. (1975) *Biochem. Biophys. Res. Commun.* **62**, 478–484

- Eppstein, D. A., Thoma, J. A., Scott, H. A. & Young, S. Y., III (1975) *Virology* **67**, 591-594
- Fairbanks, T. L., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606-2617
- Fish, W. W., Mann, K. G. & Tanford, C. (1969) *J. Biol. Chem.* **244**, 4989-4994
- Fish, W. W., Reynolds, J. A. & Tanford, C. (1970) *J. Biol. Chem.* **245**, 5166-5168
- Gelman Instrument Co. (1968) *Gelman Procedures, Techniques and Apparatus for Electrophoresis*, Ann Arbor
- Good, T. A. & Bessman, S. P. (1964) *Anal. Biochem.* **9**, 253-262
- Goodman, J. W. (1967) *Immunochemistry* **6**, 139-149
- Halvorson, H. R. & Ackers, G. K. (1974) *J. Biol. Chem.* **249**, 967-973
- Hedrick, J. L. & Smith, A. J. (1968) *Arch. Biochim. Biophys.* **126**, 155-164
- Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 199-203
- Kober, P. A. (1917) *J. Am. Chem. Soc.* **39**, 944-949
- Kozlov, E. A., Sidorova, N. M. & Serebryani, S. B. (1975a) *J. Invertebr. Pathol.* **25**, 97-101
- Kozlov, E. A., Levitina, T. L., Sidorova, N. M., Radavski, Y. L. & Serebryani, S. B. (1975b) *J. Invertebr. Pathol.* **25**, 103-107
- Krywienzyk, J. & Bergold, G. H. (1961) *J. Insect Pathol.* **3**, 15-28
- Kuzin, A. M. & Krzehova, R. V. (1948) *Biokhimiya* **13**, 523-529; (1949) *Chem. Abstr.* **43**, 3051f
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Mukerjee, H. & Sri Ran, S. (1969) *Anal. Biochem.* **8**, 393-394
- Munoz, J. J. (1959) *Anal. Chem.* **31**, 981-985
- Neville, Jr., D. M. (1971) *J. Biol. Chem.* **246**, 6328-6334
- Ouchterlony, Ö. (1968) *Handbook of Immunodiffusion and Immuno-electrophoresis*, Ann Arbor Science Publishers, Ann Arbor
- Padhi, S. B., Eikenberry, E. F. & Chase, T., Jr. (1974) *Intervirology* **4**, 333-345
- Parish, C. R. & Marchalonis, J. J. (1970) *Anal. Biochem.* **34**, 436-450
- Pusztai, A. (1966) *Biochem. J.* **101**, 265-273
- Pusztai, A. & Watt, W. B. (1970) *Biochim. Biophys. Acta* **214**, 463-467
- Rapport, I. & Zaitlin, M. (1970) *Virology* **41**, 208-217
- Rosen, H., Bernard, C. & Levenson, S. (1962) *Anal. Biochem.* **4**, 213-221
- Schachman, H. K. (1957) *Methods Enzymol.* **4**, 32-103
- Schneider, W. C. (1957) *Methods Enzymol.* **3**, 680-684
- Scott, H. A. & Young, S. Y., III (1973) *J. Invertebr. Pathol.* **21**, 315-317
- Smith, K. M. (1967) *Insect Virology*, pp. 8-40, Academic Press, New York
- Spiro, M. J. (1973) *J. Biol. Chem.* **248**, 4446-4460
- Spiro, R. G. (1966) *Methods Enzymol.* **8**, 3-26
- Steers, E., Cravin, G. R. & Anfinson, C. B. (1965) *J. Biol. Chem.* **240**, 2478-2484
- Storer, G. B., Shepherd, M. G. & Kalmakoff, J. (1974) *Intervirology* **2**, 87-94
- Summers, M. D. & Egawa, K. (1973) *J. Virol.* **12**, 1092-1103
- Summers, M. D. & Smith, G. E. (1975) *J. Virol.* **16**, 1108-1116
- Szuchet, S. & Yphantis, D. A. (1973) *Biochemistry* **12**, 5115-5127
- Teller, D. C. (1973) *Methods Enzymol.* **27**, 346-441
- Tung, J. S. & Knight, C. A. (1972) *Anal. Biochem.* **48**, 153-163
- Waehneltdt, T. V. & Mande, P. (1970) *FEBS Lett.* **9**, 209-212
- Warren, L. (1954) *J. Biol. Chem.* **234**, 1971-1975
- Weber, K. & Kuter, D. J. (1971) *J. Biol. Chem.* **246**, 4504-4509
- Weber, K., Pringle, J. R. & Osborne, M. (1972) *Methods Enzymol.* **26**, 3-25
- Weiner, A. M., Platt, T. & Weber, K. (1972) *J. Biol. Chem.* **247**, 3242-3251
- Williams, C. & Chase, M. (1971) *Methods Immunol. Immunochem.* **3**, 103-168
- Wrigley, C. W. (1971) *Methods Enzymol.* **22**, 559-564
- Yamufugi, K., Yoshihara, F. & Hirayama, K. (1957) *Enzymologia* **19**, 53-58
- Yphantis, D. A. (1964) *Biochemistry* **3**, 297-317
- Zacharius, R., Zell, T., Morrison, J. H. & Woodlock, J. J. (1969) *Anal. Biochem.* **30**, 148-152
- Zimmerman, J. K. (1974) *Biochemistry* **13**, 384-389
- Zimmerman, J. K. & Ackers, G. K. (1971) *J. Biol. Chem.* **246**, 7289-7292