Comparison of the Structure and Polypeptide Composition of Three Double-Stranded Ribonucleic Acid-Containing Viruses (Diplornaviruses): Cytoplasmic Polyhedrosis Virus, Wound Tumor Virus, and Reovirus

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Iodination of reovirus, cytoplasmic polyhedrosis virus (CPV), and wound tumor virus (WTV), and their respective subviral forms, followed by analysis of the labeled polypeptides by using polyacrylamide gel electrophoresis, has been used to compare the protein contents of these three diplornaviruses. This approach, when combined with electron microscopy and buoyant density determinations, appears capable of localizing individual polypeptides in some of the viral and subviral forms. CPV ($\rho = 1.435 \text{ g/cm}^3$) seems to resemble reovirus cores ($\rho = 1.440 \text{ g/cm}^3$) in both ultrastructure and polypeptide composition. CPV is composed of five polypeptides with molecular weights of about 151,000, 142,000, 130,000, 67,000, and 33,000. The polyhedral matrix, which in nature encapsulates the virions, is, in turn, composed mainly of two polypeptide species with molecular weights of about 30,000 and 20,000, and several minor proteins. The proteins of WTV consist mainly of four species of polypeptide with molecular weights of about 156,000, 122,000, 63,000, and 44,000, and several minor components. These molecular weight determinations are consistent with the hypothesis that, as has been suggested for reovirus, the viral proteins of CPV and WTV seem to be coded for by monocistronic messenger RNA molecules transcribed from distinct segments of the double-stranded RNA viral genomes.

In previous communications it was observed that the structure of the nucleic acid of the insect pathogen, cytoplasmic polyhedrosis virus (CPV), was similar to that of the mammalian reovirus, in that both genomes were composed of 10 distinct double-stranded ribonucleic acid (dsRNA) segments present in equimolar amounts within the respective virions (15, 20). Furthermore, these viruses, as well as a third virus-containing segmented dsRNA (12), the plant oncogenic virus, namely, wound tumor virus (WTV), contain enzymatic activities in their superstructures capable of transcribing their respective dsRNA genomes into discrete corresponding single-stranded RNA messenger molecules (3, 4, 13, 22).

The common presence of these two properties, a segmented dsRNA genome and a virus-associated transcriptase, suggests that, despite their rather diverse host systems, these dsRNA viruses (the nomenclature diplornaviruses has been suggested by Verwoerd [31]) share the same basic mode of replication.

In the present study we have continued the structural comparison of reovirus, CPV, and WTV with special emphasis on the number and location of specific polypeptides. The combined techniques of electron microscopy and the *in vitro* labeling of proteins with ^{125}I (19) have been used for this purpose. Iodination studies with influenza virus have recently been used to suggest

that the rate of such in vitro labeling of viral polypeptides can be related to the position of the individual polypeptide on the surface of the virus (26).

MATERIALS AND METHODS

Chemicals. Sodium ^{125}I (250 mCi/ml) in 0.1 M NaOH was obtained from New England Nuclear Corp., Boston, Mass. Chloramine-T and sodium-*m*-bisulfite were obtained from Eastman Kodak Co., Rochester, N.Y. Sodium dodecyl sulfate (SDS) was from Sigma Chemical Co., St. Louis, Mo. α -Chymotrypsin was obtained from Worthington Biochemical Corp., Freehold, N.J. Electrophoresis grade acrylamide, methylene bisacrylamide, ammonium persulfate, and *N,N,N',N'*-tetramethylethylenediamine were obtained from BioRad Laboratories, Richmond, Calif. All other chemicals were reagent grade.

Preparation of viruses. Reovirus type III was grown in L cells and purified by the method of Watanabe et al. (33). Reovirus cores were prepared following the procedure of Smith et al. (25). CPV was grown in *Bombyx mori*, and both polyhedral bodies and virions were purified as described in an earlier report (13). WTV-infected tumor root tissue from sweet clover (*Melilotus alba*) and virus-infected plant cuttings for vegetative propagation were kindly provided by L. M. Black. Virus was extracted and purified as described by Kimura (3) with three exceptions. Exposure of the tumor tissue to carbon tetrachloride was eliminated, virus was concentrated by sedimentation onto a 65% sucrose- D_2O cushion in place of pelleting, and virus was purified through two cycles on 30 to 60% linear sucrose gradients.

Equilibrium density centrifugation was performed using preformed gradients of CsCl in standard buffer and ranging in densities from 1.453 to 1.25 g/cm³. Sedimentation was at 36,000 rev/min in an SW50.1 rotor for 9 hr at 4 C. Fractions were collected and refractive indices were determined on a Zeiss refractrometer. Buoyant density values were calculated according to the formula, $\rho_{\text{CsCl}} = 10.860n_d25C - 13.4974$ (32).

Electron microscopy. Samples for negative staining were dialyzed against 0.01 M ammonium acetate, pH 7.0, mixed with an equal volume of 2% phosphotungstic acid (PTA) (pH 7.0), and sprayed with a nebulizer onto a platinum, 7-hole Siemens grid which had a carbon-covered collodion film. For shadowing, the samples were sprayed on collodion-covered, 200-mesh copper grids, air dried, and uranium shadowed. For thin-sectioning, samples were fixed in 2% glutaraldehyde, postfixed in osmium, embedded in epoxy, and sectioned with the LKB ultratome. Sections were poststained, first with 20% uranyl acetate in methanol for 20 min, followed by lead citrate for 5 min, and examined in a Siemens Elmiskop I electron microscope.

Dissociation of virus. Samples were dissociated either prior to, or after, iodination. In the former case, CPV and WTV samples in buffer were dissociated with 1% SDS at 100 C for 2 min, whereas reovirus was dissociated with 1% SDS at 100 C for 2 min, or

with 0.2% SDS and 6 M urea at 45 C for 15 min. For dissociation of iodinated CPV and WTV, samples were exposed to 1% SDS, 6 M urea, 0.03 M dithiothreitol (DTT), 0.1 M tris(hydroxymethyl)amino-methane (Tris), pH 7.5, at 100 C for 2 min. Iodinated reovirus was dissociated in 0.6% SDS, 6 M urea, 0.007 M DTT, and 0.007 M phosphate buffer, pH 7.0, at 37 C for 1 hr. Unlabeled CPV used for molecular weight determinations in Fig. 8 was disrupted in 1% SDS, 0.014 M DTT, and 0.006 M phosphate buffer, by heating at 45 C for 20 min.

In vitro iodination. The tyrosine residues of the samples under study were iodinated by using chloramine-T as the oxidizing agent. The reaction mixture consisted of 50 μliters of virus or subviral component, 35 μliters of 0.14 M Tris-hydrochloride, pH 7.5, and 5 μliters of Na^{125}I in 0.2 M Tris, pH 7.5 (200 μCi /sample). Labeling was initiated by addition of 20 μliters of chloramine-T (10 mg/ml) and was allowed to continue at room temperature for the various times listed in the figure legends. The reaction was terminated by addition of 20 μliters of sodium-*m*-bisulfite (24 mg/ml) and 1 μliter of 0.01 M potassium iodide. Excess ^{125}I was removed by overnight dialysis with several changes of buffer (0.01 M Tris-hydrochloride, pH 7.5, 0.001 M ethylenediaminetetraacetic acid [EDTA], 0.1 M NaCl for intact virus, and 0.01 M Tris-hydrochloride, pH 7.5, 0.001 M EDTA, 0.1% SDS for dissociated virus). When polyhedral bodies were iodinated, excess ^{125}I was removed by sedimentation through 30% sucrose in an SW65 rotor at 10,000 rev/min for 15 min at 4 C. The pellet was resuspended in 5 ml of buffer (0.1 M Tris-hydrochloride, pH 7.5, 0.001 M EDTA, 0.1 M NaCl) and resedimented in a Sorvall refrigerated centrifuge at 10,000 rev/min for 10 min. This final pellet of ^{125}I -labeled polyhedral bodies was solubilized with 1% SDS-8 M urea at 100 C for 2 min prior to electrophoresis.

Polyacrylamide gel electrophoresis. Dissociated viral fractions were made 5% in sucrose, 0.005% in bromophenol blue dye, and analyzed on 5% polyacrylamide gels (120 mm in length) prepared in 0.1 M sodium phosphate buffer, pH 7.2, 10% glycerol, 0.13% bisacrylamide, 0.02 M EDTA, 0.1% SDS, 0.1% temed (*N,N,N',N'*-tetramethylethylenediamine), 0.08% persulfate. Electrophoresis buffer consisted of 0.1 M sodium phosphate, pH 7.2, 0.1% SDS, 0.02 M EDTA. Electrophoresis was usually done at 6 ma per gel for 25 hr or until the dye front was near the bottom of the gel. For determination of ^{125}I content, gels were frozen in ethanol-dry ice and fractionated by a mechanical gel fractionator into 1-mm slices. The gel fractions were dried and counted in a toluene base scintillation fluid (4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2-(5-phenyloxazolyl)benzene per liter of toluene) in an Intertechnique liquid scintillation counter at full window. Molecular weight determination of polypeptides was done following the general procedure of Weber and Osborn (35). Polypeptide staining was performed with Coomassie brilliant blue (0.25%) in a mixture of 50% methanol-10% glacial acetic acid for 3 hr at room temperature. Gels were allowed to remain in destaining solution (7.5% glacial acetic

acid-5% methanol) for 1 hr at room temperature prior to electrophoretic destaining in the same solution.

RESULTS

Reovirus: efficacy of iodination as a means of localizing virus polypeptides in relation to virus surface. Studies with reovirus type III (25) have shown that reoviral polypeptides can be isolated from three sources: whole virions, viral "top component" (RNA-free viral shells), and subviral core particles obtained by enzymatic removal of the outercoat polypeptides. This same report has shown that reovirus polypeptides are distributed in three classes, λ (containing two components, λ_1 and λ_2), μ (containing two components, μ_1 and μ_2), and σ (containing three components, σ_1 , σ_2 , and σ_3). Because it is already known which of the reovirus type III polypeptides compose the external coat and which the internal core proteins (25, 16), this system seemed to provide an excellent control to our general proposal to examine the position of diplornavirus polypeptides by using the chloramine-T iodination technique.

Equilibrium sedimentation and electron microscopy of reovirus. When a crude preparation of reovirus type III was sedimented to equilibrium in preformed gradients of CsCl, the sample separated into three major areas of light-scattering particles, a dense major band, two less dense

satellite bands, and a third series of even less dense minor bands. These three areas were collected and repurified on separate gradients of CsCl. Based on buoyant density determinations of 1.38, 1.36, and 1.30 g/cm³, the respective bands seen in Fig. 1 were arbitrarily designated V, S, and T to represent virions, satellite virions, and top component. Representative samples of these three preparations were then examined in the electron microscope. Figure 2 shows that, although both populations S and V are approximately 75 nm in diameter and morphologically similar icosahedrons, the satellite virions ($\rho = 1.36$ g/cm³, Fig. 2a) have uniformly allowed little penetration of stain, whereas the major virion fraction ($\rho = 1.38$ g/cm³, Fig. 2b) shows heavy penetration by PTA. Top component material ($\rho = 1.30$ g/cm³, Fig. 2c) showed empty intact shells which were completely permeable to PTA and composed primarily of an inner shell membrane and a relatively intact outer capsid structure. Upon storage at 4 C, these shells tended to degrade into intact shell membranes and random aggregates of capsomeres (Fig. 2d), while both populations S and V appeared to remain intact.

Iodination and gel electrophoresis of the polypeptides of reovirus and its subviral particles. Samples of these three populations were then dissociated with urea-SDS, labeled with ¹²⁵I,

FIG. 1. Sedimentation of reovirus subfractions on a preformed gradient of CsCl centrifuged for 9 hr in an SW50.1 rotor at 36,000 rev/min. V, Virion ($\rho = 1.38$ g/cm³); S, satellite virion ($\rho = 1.36$ g/cm³); T, top component ($\rho = 1.30$ g/cm³).

FIG. 2. Electron micrographs of reovirus satellite (a), reovirus virion (b), top component (c), top component stored at 4°C (d), and reovirus core (e). Particles shown in a-d were isolated from bands of CsCl equilibrium density gradients as shown in Fig. 1, dialyzed versus 0.01 M ammonium acetate, pH 7.0, negatively stained with 1% PTA at pH 7.0, and sprayed with a nebulizer onto a 7-hole, platinum Siemens grid which had a carbon-coated collodion film. Particles shown in (e) were treated in 0.01 M ammonium acetate, pH 7.0, with 100 μ g of α -chymotrypsin/ml at 37°C for 60 min prior to negative staining. a-d, $\times 160,000$; e, $\times 260,000$.

and subjected to polyacrylamide gel electrophoresis. Figure 3a shows that the three major classes of viral polypeptides, λ , μ , and σ , and the minor peptide component VIII are susceptible to iodination. The distribution of 125 I label is 21, 38, 36, and 4%, respectively, and agrees favorably with the values of 24, 40, 32, and 4% obtained when virus is labeled in vivo with either 14 C or 3 H amino acids (25). This distribution pattern remains constant within those time periods tested, from 1 to 15 min. Though not diagrammed, no major differences could be detected in the polypeptide compositions of virion, satellite, or top components. This finding, together with the inability to detect any gross differences in total nucleic acid content between the two populations

V and S (Lewandowski and Traynor, *unpublished observations*), suggests that the increased buoyant density and susceptibility to PTA uptake of the majority population may be due to loosening rather than removal of surface polypeptides. Such a realignment would explain why populations of particles with the same RNA-protein ratio exhibit differential uptake and binding of small molecules like CsCl (21), and PTA.

Previous results suggested to us a potential relationship between reovirus core particles and purified CPV. Reovirus cores and CPV possess similar segmented dsRNA genomes of approximately 15×10^6 daltons (14), similar buoyant densities in CsCl, 1.435 ± 0.005 g/cm 3 (25, 15), and similar transcriptase activities demonstrable

FIG. 3. *a*, Polyacrylamide gel electrophoresis of reovirus dissociated with 6 M urea-0.2% SDS at 45°C for 15 min prior to iodination. Two hundred μ liters of sample was iodinated for 15 min at room temperature as described in Materials and Methods. The iodinated sample was analyzed on a 5% polyacrylamide gel. Migration was for 6 hr at 3 mA per tube, and 1-mm slices were examined for their radioactive content. Direction of migration is from left to right. *b*, Reovirus core particle (Δ) and satellite core ($\bullet$), dissociated

in vitro (4, 13, 22, 24). Accordingly, intact reovirus was digested with chymotrypsin and sedimented to equilibrium in CsCl. Two nucleoprotein core components resulted, a major one at the density of 1.44 g/cm³ (Fig. 2e) and a minor satellite component at the density of 1.42 g/cm³. Little difference could be observed between these two populations by electron microscopy. The most striking physical feature of reovirus cores is the presence of regularly spaced, chimney-like spike projections (Fig. 2e; reference 18) on the 12 fivefold vertices of the icosahedron. The two core populations were disrupted with urea-SDS, labeled with ^{125}I , and analyzed for their polypeptide content by polyacrylamide gel electrophoresis. The superimposed patterns are presented in Fig. 3b. In agreement with the literature data (16, 18), reovirus core consists mainly of the λ polypeptides, and polypeptide σ_2 , and a minor amount of μ_1 . Satellite core, however, has additional labeled material migrating between the μ and σ peaks, and as low-molecular-weight peptide. Satellite core apparently represents an incomplete stage of removal of the discrete degradation products X, Y, and Z (37), and small peptides of the major coat polypeptides μ and σ_3 , thereby causing the core particles to exhibit a decreased buoyant density.

Localization of reovirus polypeptides. We next investigated whether one could use the ^{125}I in vitro labeling procedure to successfully discriminate between surface and internal polypeptides in the intact virion by the rate of labeling of the tyrosines of each class of polypeptide. Intact reovirus type III was treated with ^{125}I reagent for 60 sec; the subsequently obtained electropherograms of the dissociated proteins are shown in Fig. 3c. Two striking features are observed: first, the λ core polypeptides contain very little label, about 7% of total compared with the 21% of total when dissociated virus was labeled with iodine (Fig. 3a), and second, the external coat polypeptides of the μ and σ classes are appreciably labeled, and, in particular, σ has a disproportionate amount of label (58%) compared to the amount observed (36%) when dissociated virus is iodinated. Thus, limiting the iodination time appears to discriminate between

with 5 M urea-0.2% SDS at 45°C for 45 min prior to iodination. Iodination was 5 min at room temperature. The iodinated samples were placed on 4% polyacrylamide gels and run for 6 hr at 3 mA per tube. *c*, Reovirus iodinated prior to dissociation with 6 M urea-0.2% SDS at 100°C for 2 min. One hundred μ liters of sample was iodinated for 60 sec as described in Materials and Methods. Electrophoresis was on 5% polyacrylamide gels for 27 hr at 7 mA per tube.

polypeptides in the undenatured virion. Not only is the technique capable of accurately confirming which classes of viral peptides comprise the exterior coat, but also which particular class (in this case σ) is most accessible to iodination and presumably, therefore, most exterior. These *in vitro* data are in good agreement with the recent data showing that, *in vivo*, σ_3 is the first protein to be removed during the uncoating process (23) followed by μ_2 . When intact reovirus cores were iodinated prior to SDS dissociation (diagram not shown) core polypeptide σ in particular showed a lower percentage of total label relative to the λ components than was seen when cores were dissociated prior to labeling (Fig. 3b). Thus, in the intact reovirus core, the tyrosines of the λ polypeptides appear to be more available for iodination than are those of the μ and σ core polypeptides.

CPV: electron microscopy of polyhedral bodies. CPV, perhaps the most widespread viral pathogen of insects (1), derives its name from light microscopy observations that infection is associated

with the formation of unique cytoplasmic structures which are designated "polyhedral inclusion bodies." Inasmuch as polyhedral bodies of certain shapes (icosahedral, hexahedral, and pyramidal [10]) can be cloned to give rise to polyhedra of that particular shape in subsequent infections, polyhedral shapes appear to be controlled by the strain of virus which the polyhedra contain.

Thin-section electron microscopy was performed on hexahedral polyhedral bodies produced in silkworm (*Bombyx mori*) larvae. Figure 4a shows the gross structure of native virions as they exist in the occlusion body prior to dissociation of the polyhedral matrix. At the edges of these polyhedra one can observe (arrows, Fig. 4a and 4b) native virions as they are partially embedded or just being released from the highly crystallized polyhedral protein matrix. The extruding virion appears to have a dense core area surrounded by an outer shell membrane which has discernible spikes but no thick outer capsid structure analogous to the reovirus coat.

Equilibrium sedimentation and electron micros-

FIG. 4. Thin-section electron micrograph of CPV polyhedral bodies and virus. Polyhedral bodies were prepared as described in Materials and Methods and fixed in 2% glutaraldehyde, postfixated in osmium, embedded in epoxy, and sectioned with the LKB ultratome. Sections were stained with 20% uranyl acetate in methanol for 20 min, poststained in lead citrate for 5 min, and examined in a Siemens Elmiskop I electron microscope. a, $\times 84,000$; b, $\times 390,000$.

copy of purified CPV. To observe the fine structure of CPV, purified polyhedral bodies were exposed to pH conditions known to solubilize the polyhedral matrix without destroying the infectivity of the released virions (13). Density gradient centrifugation of such concentrated extracts on preformed gradients of CsCl produced three detectable light-scattering components at equilibrium. A typical light-scattering pattern (Fig. 5) shows the major virus band (V) at $\rho = 1.435 \text{ g/cm}^3$, a minor diffuse shoulder area (S) at 1.425 g/cm^3 , and a very sharp minor top band (T) at $\rho = 1.30 \text{ g/cm}^3$. Previous work in our laboratory (15) has shown that the major band (V) is composed of virions containing the entire genome complement of 10 dsRNA segments in equimolar amounts, whereas the diffuse shoulder area contains satellite virions (S) which are deficient in some of the smaller RNA segments. By analogy to reovirus, the RNA-free material equilibrating at $\rho = 1.30 \text{ g/cm}^3$ was designated T for top component.

When analyzed by electron microscope, particles from the major virus band were by no means homogeneous with respect to their response to PTA staining. This variation in uptake of stain was at times so striking that we selected several micrographs (Fig. 6) which highlight different structural features of CPV. Virions appear to be icosahedrons with an average diameter of approximately 65 nm, a capsomeric-like surface structure, the suggestion of a nearly six-sided outline with 20 capsomeres often visible along the circumference, and distinct pyramidal spikes on the corners of the polygon (Fig. 6a and 6b). These pyramidal spikes which seem to originate deep within the central core area of the virus are particularly prominent in those particles which are heavily internally stained and are distinct from the chimney-like spikes seen with reovirus cores (Fig. 2e). Though six spikes are readily discernible, one could predict, based on the observed structure of the virion, a total of either 10, or, more probably, 12 (9). Frequently, virions (Fig. 6c) seem to be extruding long, tail-like structures. Due to their 6 to 7 nm diameter, it is unlikely that these tails are single RNA duplexes which would measure approximately 2 nm. More likely, these structures represent duplex RNA bundles or nucleoprotein structures somewhat like those of eucaryotic chromosome fibers (6). When virus samples are given minimum electron beam exposure (36), one can observe the capsomeric-like surface structures but not the spike structures in greater detail (Fig. 6d).

In general, no difference was observed between the structure of virions banding at 1.435 g/cm^3 and particles in the less dense, diffuse satellite

FIG. 5. Equilibrium sedimentation of CPV on a preformed gradient of CsCl ($\rho = 1.370$ to 1.453 g/cm^3) for 9 hr at 36,000 rev/min in an SW50.1 rotor. V, Virion ($\rho = 1.435 \text{ g/cm}^3$); S, satellite virion ($\rho = 1.425 \text{ g/cm}^3$); T, top component ($\rho = 1.30 \text{ g/cm}^3$).

zone, except that satellite virions more frequently show tail-like structures protruding from virions. Exposure of CPV to chymotrypsin under the same conditions that produce reovirus cores (25)

FIG. 6. Electron micrographs showing various morphological aspects of CPV isolated from CsCl density gradients as shown in Fig. 5. *a*, Virions with negative staining; *b*, shadowed virion; *c*, virion extruding nucleic acid containing tails; *d*, negatively stained virions given minimum electron beam exposure; and *e*, negatively stained top component. *a*, $\times 215,000$; *b*, $\times 160,000$; *c*, $\times 95,000$; *d* and *e*, $\times 320,000$.

from whole reovirus showed no gross effect on either CPV ultrastructure or buoyant density. Electron microscope examination of the material banding at a density of 1.30 g/cm^3 shows empty virions completely permeable to PTA (Fig. 6*e*) and, as was true of the RNA-free uppermost reovirion band, aggregates of partially dissociated viral shells.

Polypeptide composition of CPV and its sub-viral forms. The polypeptide components of CPV were investigated by the same techniques employed in model experiments with reovirus. Samples of the three viral components, virions, satellite area, and top component, were first dissociated with urea-SDS, labeled with ^{125}I , and subjected to analysis on polyacrylamide gels. The

results are seen in the electropherograms of Fig. 7. Disrupted whole virions (Fig. 7a) were found to contain five distinct iodine-labeled polypeptides and about 10% of total ^{125}I migrating as small peptide material in front of the dye marker (insert). The proportion of total radioactivity in each of the viral polypeptides obtained after iodination of dissociated whole virus was: I, 36%; II, 16%; III, 34%; IV, 2%; and V, 10%, respectively.

When dissociated satellite and dissociated top component of CPV were subsequently labeled *in vitro* and analyzed on parallel polyacrylamide gels, the polypeptide patterns shown in Fig. 7b and 7c were observed. Attention might be directed to the occurrence of polypeptide breakdown as indicated by the presence of high background label migrating between components III and V (Fig. 7b). This breakdown phenomenon is also apparent in the analysis of dissociated top component (Fig. 7c). Because the size distribution of

specific breakdown products of the larger species might well overlap several of the native species in such a multicomponent system, it is generally rather difficult to relate breakdown products to a particular native component. The exception, however, seems to be breakdown product I₁ which, because of its size, can only be derived from component I. The release of the dsRNA of CPV, resulting first in a satellite area deficient in a portion of the RNA (15) and subsequently in the fraction called top component which, by virtue of its low buoyant density, is apparently devoid of any of the RNA genome, seems therefore to be accompanied by the breakdown particularly of the largest polypeptide species, component I.

Localization of CPV polypeptides. The rate at which the polypeptides of intact CPV virions are iodinated was determined in the same manner as with reovirus. The electrophoretic pattern of labeled polypeptides obtained when CsCl-puri-

Fig. 7. Electropherograms of iodinated polypeptides of CPV virion (V), satellite virion (S), and top component (T) isolated from preformed gradients of CsCl (Fig. 5). Samples in a to c were dialyzed against 0.1 M Tris-hydrochloride (pH 7.5)-0.001 M EDTA-0.1 M NaCl, dissociated with 1% SDS, and iodinated for 60 sec as described in Materials and Methods. The sample in d was iodinated for 60 sec prior to dissociation. Electrophoresis was on 5% polyacrylamide gels for 25 hr at 6 mA per tube. The insert in a was included to demonstrate the presence of small peptide material which migrates ahead of the dye marker.

fied whole virus was iodinated for a brief period prior to dissociation is shown in Fig. 7d. Component III has a higher portion (56%), whereas components II, IV, and V have lower portions (5%, 1%, and 2%, respectively) of total label than when whole virus is dissociated prior to iodination (Fig. 7a). Component I (34%) is slightly lower. Components I and III may therefore represent outer, and components II, IV, and V, inner polypeptides of the CPV particle. It must be understood, however, that the specific location of the tyrosine residues in the individual viral polypeptides is not known. Thus, the possibility that tyrosine groups of the surface polypeptides are buried or those of internal polypeptides are exposed cannot yet be eliminated in any interpretation by using this technique of ^{125}I labeling of intact virus or subviral particles.

Molecular weight determinations of CPV polypeptides. Molecular weights of the polypeptides from CPV were determined by comparison of their electrophoretic mobilities on SDS gels with those of proteins of known molecular weights (35); polypeptides used as standards were tektin a' and a (220,000 and 200,000); myosin (200,000); *Escherichia coli* RNA polymerase subunits β' and β (163,000 and 155,000); gamma globulin H_2L_2 (150,000); β -galactosidase (130,000); bovine serum albumin (67,000); gamma globulin H chain (50,000); ovalbumin (45,000); gamma globulin L chain (22,000); and tobacco mosaic virus (TMV) coat protein (17,500). Figure 8a illustrates the comparative migrations of CPV and markers on 5% polyacrylamide gels. When plotted as in Fig. 8b, these data show that, in the gel system used, migration is linear within the 220,000 to 130,000 range and again within the 110,000 to 18,000 molecular weight ranges. The molecular weights of CPV polypeptides I through V, determined from the plots of Fig. 8b, are 151,000, 142,000, 130,000, 67,000, and 33,000, respectively. Break-down product I_1 seems to have resulted from the cleavage of a fragment(s) of 5,000 to 6,000 molecular weight from polypeptide I.

An incidental point arising from these determinations of molecular weights concerns the λ_1 and λ_2 polypeptides of reovirus type III. Literature values place these two species at 155,000 and 140,000, a difference of 15,000 (25). We have, however, consistently observed a difference of only approximately 6,000 (Fig. 8a).

Recently it has been shown that electrophoretic mobilities in SDS gels can be affected not only by changes in molecular size but also by changes in charge and molecular conformation (29) caused by the chemical modification of polypeptides. To determine whether the iodination procedure alters

the apparent molecular weights of the viral polypeptides, iodinated CPV polypeptides were subjected to co-electrophoresis in the same gel with unlabeled carrier polypeptides (figure not shown). Examination of the individual slices for both dye stain and ^{125}I content showed that the iodinated polypeptides migrated, on the average, 5% more slowly than nontreated proteins. Consequently, whenever molecular weight values were determined in subsequent studies by using iodinated proteins versus non-iodinated markers, a 5% correction factor was applied.

Once the molecular weights of the CPV polypeptide were determined, it was of interest to see if, as appears to be the case for reovirus (37), a clear correspondence exists between the size of the dsRNA segments and the size of the discrete viral polypeptides. The genome of CPV consists of 10 discrete dsRNA segments ranging in size from the largest, estimated at 2.7×10^6 daltons, to the smallest, at 0.37×10^6 daltons (14). It would appear (Table 1) that viral polypeptides I through V could be coded for by dsRNA segments I, II, III, VI, and VIII, respectively. This leaves, however, five genome segments either without a corresponding viral structural protein or coding for proteins which might be present in the virus in amounts sufficiently small to be undetectable by either dye retention or ^{125}I labeling.

Analysis of the polyhedral proteins of CPV.

We have already noted that the shape of the polyhedral body seems to be determined by the strain of virus which it contains. It seemed possible, therefore, that the highly organized polyhedral matrix (Fig. 4b) is composed of proteins which are also direct gene products of the viral genome. To investigate this possibility, polyhedral bodies were purified on a step-gradient of sucrose (13) and iodinated. The bulk of the polyhedral body appears, by microscopy, to be material other than virions-proper (Fig. 4a). Hence it seemed reasonable to assume that the pattern of in vitro labeling would reflect the high content of the polyhedral polypeptide(s). Iodinated polyhedral bodies were separated from free, nonreacted ^{125}I by several cycles of sedimentation through step-gradients of sucrose and were solubilized with urea-SDS. The labeled protein was then analyzed by polyacrylamide gel electrophoresis in the presence of unlabeled viral marker polypeptides. The results are seen in Fig. 9.

The labeling pattern would suggest that polyhedral protein consists primarily of two major polypeptides with approximate molecular weights of 29,500 and 19,500. There are, however, minor amounts of labeled protein in discrete positions,

FIG. 8. Molecular weight determinations of CPV polypeptides based on electrophoretic migration of assorted marker species. *a*, Samples were solubilized with 1% SDS and subjected to electrophoresis on 5% polyacrylamide-0.1% SDS gels at 8 mA per gel for 17.5 hr. Abbreviations: Ta' and Ta , tektin a' and a ; M , myosin; β' and β , *E. coli* RNA polymerase subunits B' and B ; H_2L_2 , gamma globulin H_2L_2 ; β -gal, β -galactosidase; SA , bovine serum albumin; H , heavy chain of gamma globulin; OA , ovalbumin; L , light chain of gamma globulin; TMV , TMV coat protein; I-V, CPV polypeptides; λ , μ , σ , reovirus polypeptides. Migration is from top to bottom, *b*, Plot of molecular weight versus distance migrated.

TABLE 1. Correspondence of genome segment size of cytoplasmic polyhedrosis virus to the size of viral and polyhedral polypeptides

CPV genome component no.	Molecular weight				
	Observed dsRNA segment ^a (× 10 ⁶)	Estimated ssRNA ^b transcript (× 10 ⁶)	Estimated gene product ^c	Poly-peptide observed in the virion	Poly-peptide observed in polyhedral matrix
I	2.70	1.35	150,000	151,000	
II	2.44 ^d	1.22 ^d	136,000 ^d	142,000	
III				130,000	
IV	2.15	1.07	119,500		122,000 ^e
V	1.85	0.92	102,800		106,000 ^e
VI	1.14	0.57	64,000	67,000	
VII	0.83	0.41	46,000		48,000 ^e
VIII	0.62	0.31	34,000	33,000	
IX	0.54	0.27	30,000		29,500
X	0.37	0.18	20,500		19,500

^a Calculated from Lewandowski and Leppa (14) by using reovirus RNA segments as molecular weight markers.

^b ssRNA, Single-stranded RNA.

^c Estimated molecular weight of gene product = mol wt ssRNA message/[mol wt nucleotide/(3 nucleotides/aa)] × mol wt aa. Mol wt nucleotide, 3 × 10²; mol wt aa, 10²; aa, amino acid.

^d Average values for components II and III which migrate closely.

^e Minor peaks indicated by arrows in Fig. 9.

some of which overlap the marker viral structural polypeptides and several of which do not. Comparison with unlabeled CPV structural protein markers and application of the 5% correction factor associated with the iodination procedure provide the molecular weight estimates seen in the last column of Table 1; the molecular weights of the two major and three of the minor polyhedral polypeptides suggest that they might be direct gene products of the five remaining cistrons of the genome of CPV. As the polypeptides of the polyhedral matrix are being laid down around individual virus particles in the cytoplasm of the host cell (27), it is possible that other gene products which, although perhaps not part of the final virion superstructure but which were functioning during virus replication and maturation, might easily become entrapped in the growing polyhedral structure. This may explain the presence of the minor peaks in Fig. 9.

WTV: Equilibrium sedimentation and electron microscopy of WTV. A relationship between reovirus and WTV has been proposed based on electron microscopy data showing somewhat similar external morphologies and on the common presence of dsRNA as the genetic material (2, 8, 28, 30). When sucrose-purified WTV was sedimented to equilibrium in preformed gradients of CsCl,

FIG. 9. Electropherogram of polypeptides from iodinated, dissociated polyhedral bodies of CPV. Polyhedral bodies were purified on sucrose gradients as described in Materials and Methods, iodinated for 15 min, and solubilized with 1% SDS-8 M urea at 100 C for 2 min. This labeled sample was mixed with unlabeled marker CPV polypeptides and subjected to electrophoresis on 5% polyacrylamide-0.1% SDS gels at 7 mA per gel for 21.5 hr. Migration is from left to right. Arrows indicate the positions of three minor polypeptide species which, in addition to the two major peaks, correspond in size to expected viral gene products (see Table I).

the preparation separated into three major classes of light-scattering particles: two lower bands which made up the bulk of the preparation, a diffuse middle area, and an upper area of obviously less dense material. Immediate resedimentation of these three areas resulted in the fractions seen in Fig. 10. The two lower bands had buoyant densities of 1.445 and 1.425 g/cm³, whereas the middle diffuse area and the upper bands equilibrated at 1.39 to 1.41 and 1.30 g/cm³, respectively. Due primarily to previous reports that the buoyant density of WTV ranges from 1.395 to 1.410 (D. R. Black, Ph.D. thesis, Univ. of California, Berkeley, Calif., 1970; reference 7), the middle diffuse area was arbitrarily designated V (for virion). Because the buoyant densities of the lower bands approximately resembled those of intact CPV and reovirus cores, these fractions were tentatively designated C (for cores), whereas the top band was designated T (for top component). The presence of upper bands (T) in the center gradient which received only the lower material (C) suggested that T could arise as a breakdown product of C.

Electron microscopy of the original sucrose-purified preparation and of the three fractions resulting from CsCl resedimentation is shown in Fig. 11. Figure 11a shows a typical sucrose-

FIG. 10. *Equilibrium sedimentation of wound tumor virion and subviral components. Sucrose-purified WTV had previously been sedimented to equilibrium in preformed CsCl gradients ($\rho = 1.453$ to 1.30 g/cm³), and three major classes of light-scattering particles resulted: two lower bands equilibrating at $\rho = 1.445$ and $\rho = 1.425$ g/cm³, termed fraction C, a middle diffuse area with a density from 1.39 to 1.41 g/cm³, termed fraction V, and an upper area equilibrating at $\rho = 1.30$ g/cm³, termed fraction T. Immediate resedimentation of these three areas resulted in the fractions seen above. All preformed gradients were 3 ml in volume with a 2 -ml sample overlay and were run at $40,000$ rev/min for 12 hr in an SW50.1 rotor.*

purified preparation containing mainly intact virions approximately 66 nm in diameter. Figure 11b was selected to show some disrupting and apparently empty virions as well as bundles of strands, possibly analogous to those seen with CPV. The virions are hexagonal, possessing an inner core area 35 to 40 nm in diameter (Fig. 11c) surrounded by an outer capsid membrane. Figure 11d, e, and f contain V, C, and T components, respectively. It is apparent that, compared to reovirus or CPV, prior exposure of WTV to cesium chloride has a strong degradative effect when virus is subsequently dialyzed to remove salts and observed by PTA negative staining. Whereas both the V and C fractions seem to have retained an internal core structure, both are now relatively permeable to PTA and display little of the highly organized capsid structure seen in the preparation not exposed to CsCl. It is not yet clear whether the loss of capsid material occurred during CsCl sedimentation or during the subsequent dialysis or PTA staining steps. Top component (Fig. 11f) is composed mainly of empty shells, completely permeable to PTA and devoid of much of its capsid structure and none of the virus-free strands seen in the V and C preparations. Exposure of WTV to chymotrypsin failed

to produce a particle with spike structures analogous to those on reovirus cores or CPV.

Polypeptide composition of WTV and its subviral forms. Samples of the sucrose preparation and fractions C, V, and T from CsCl were then dissociated with SDS, labeled with ¹²⁵I, and analyzed on polyacrylamide gels. The resulting electropherograms appear in the upper portions of the four panels of Fig. 12. Since the $\sim 16 \times 10^6$ daltons of viral genome comprise 22% of total virion mass (12), we can calculate that total protein mass is about 60×10^6 daltons per virion. With the sucrose gradient-purified mixture, the ¹²⁵I content of this protein mass is distributed between two major polypeptide peaks (II and IV), two small peaks (I and III), and several other peaks not much greater than the background (arrows, Fig. 12a). With the V fraction from CsCl, the number and height of these background peaks were considerably increased, but again peaks II and IV predominated (Fig. 12c). Despite the appearance in Fig. 11e of a loss of some of the outer capsomere structure, the polypeptide content of fraction C_{II} ($\rho = 1.425$ g/cm³) shown in Fig. 12b is very similar to that of sucrose-purified WTV seen in Fig. 12a. Though not shown, the polypeptide pattern of fraction C_I ($\rho = 1.445$

FIG. 11. Electron micrographs of WTV, prepared and stained as in Fig. 2. *a* and *b*, WTV purified on sucrose density gradients by the Kimura modification as described in Materials and Methods; *c*, WTV partially disrupting to reveal an inner core-like area. Rods of potato virus X were added to enhance drop spreading. *d-f*, Viral components from CsCl density gradient centrifugation (Fig. 10); *d*, component V; *e*, component C; *f*, component T. *a-f*, $\times 160,000$.

FIG. 12. Electropherogram of iodinated WTV polypeptides. Upper portions of each part show viral fractions dissociated with 1% SDS for 2 min at 100 C prior to iodination for 10 min. Lower portions of each panel show the same viral fractions iodinated intact for 30 sec prior to dissociation. A, Sucrose-purified WTV. Unlabeled CPV was added to this sample to serve as marker, and the stained pattern is seen at the top of the figure. Arrows indicate the migration positions which would correspond to those of expected viral gene products (see Table 2). b, WTV "C_{II}" band isolated from CsCl at the equilibrium position of $\rho = 1.425$ g/cm³. c, WTV "V" area isolated from CsCl at the equilibrium position of $\rho = 1.410$ to 1.390 g/cm³. d, WTV "T" band isolated from CsCl at the equilibrium position of $\rho = 1.30$ g/cm³.

g/cm³) was also much like that of fraction C_{II}. This suggests that the apparent loss of capsomeres in all the subfractions of CsCl-exposed WTV (Fig. 12b-d) may not have occurred during sedimentation itself but rather as a result of dialysis against low salt buffer or PTA staining prior to microscopy of the CsCl-exposed sample.

Analysis of the polypeptide constituency of WTV top component (T) is seen in the upper portion of Fig. 12d. In this case, no distinct peaks are discernible at positions I and III. Since the bulk of the top component appears to be composed of empty shells, the polypeptide pattern in Fig. 12d would suggest that components II and IV probably comprise the membrane and

capsid polypeptides of these nucleic acid-free shells.

Location and molecular weight determinations of WTV polypeptides. To investigate the locations of the viral polypeptides, samples of the sucrose and CsCl fractions of WTV were labeled prior to dissociation and analyzed in parallel. The lower halves of Fig. 12a-d show that, in all cases, the polypeptide most susceptible to iodination in the intact structures was species II. This tentatively suggests that polypeptide II comprises the outer capsomeric layer of WTV and its CsCl subfractions. Of the non-SDS-treated samples, the tyrosine residues of polypeptide IV were available for iodination only in top component (T), and then

TABLE 2. Correspondence of genome segment size of wound tumor virus to the size of virus-associated polypeptides

WTV genome component no.	Molecular weight			
	Observed dsRNA segment ^a (× 10 ⁶)	Estimated ssRNA ^b transcript	Estimated gene product ^c	Polypeptide observed in the virion
I	2.80	1.40	155,000	156,000
II	2.30	1.15	128,000	122,000
III	2.08	1.04	116,000	
IV-V	1.72	0.86	96,000	
VI-VII	1.08	0.54	60,000	63,000
VIII	1.01	0.50	55,500	
IX	0.77	0.38	42,000	44,000
X-XI	0.56	0.28	31,000	
XII	0.54	0.27	30,000	
XIII	0.33	0.16	14,500	

^a Calculated from Lewandowski and Leppa (14) by using reovirus RNA segments as molecular weight markers.

^b ssRNA, Single-stranded RNA.

^c See footnote b in Table 1.

only partially (Fig. 12d). The interpretation of the data obtained with WTV seems to be difficult, since the virus is unstable to CsCl.

Molecular weight determinations of these labeled polypeptides were made by including unlabeled CPV in several gels. The migration of the stained CPV markers is diagrammatically presented at the top of Fig. 12a. Based on this study, the following values were assigned to the four distinct peaks of WTV polypeptide label: I, 156,000; II, 122,000; III, 63,000; and IV, 44,000. These molecular weight estimates are recorded in Table 2, which shows that a reasonable correspondence exists between the size of those polypeptides present in the virion and the size of several of the estimated gene products. These latter values are derived from data (14) which suggest that the genome of WTV is composed of 13 dsRNA segments which are electrophoretically separable into 10 distinct classes. One can estimate that viral polypeptides I through IV might be coded for by dsRNA segments, I, II(III), VI(VII), and IX. The predicted electrophoretic positions of the remaining estimated gene products have been designated by arrows in Fig. 12a. It is not yet possible, however, to determine if the minor peaks of polypeptide label in and around these positions correspond to direct viral gene products.

DISCUSSION

When the genome of a dsRNA-containing virion is introduced into a host cell, the informa-

tion which the viral RNA contains cannot be translated directly by the ribosomes; transcription of single-stranded messages is first required. The RNA extracted from dsRNA viruses does not appear to be infectious. Transcription of the input genome therefore does not appear to be accomplished by any enzyme pre-existing in the uninfected cell, but rather by the RNA-dependent RNA polymerase introduced along with the respective templates. It would seem to follow that translation of these messenger RNA species then provides the remaining virus proteins required for successful multiplication of the virion.

Viral genome-specified polypeptides fall into two categories, those which compose the superstructure of the virion and nonstructural polypeptides which function in virus-specified biosynthesis. In the case of the dsRNA-containing viruses, these two categories appear to overlap.

Whereas each reovirion has a chymotrypsin-digestible outer coat structure composed mainly of two proteins, μ_2 and the outermost component σ_3 , with CPV this role of protective outer coat seems to be borne on a collective basis by the polyhedral matrix, also composed mainly of two proteins. The polypeptides most susceptible to iodination with the intact viral or subviral forms are μ_2 and σ_3 for intact reovirus, the λ components for reovirus cores, components I and III for CPV, and class II polypeptide for WTV. Unlike intact reovirus, purified WTV and CPV show no detectable response to exposure to chymotrypsin.

All attempts in this laboratory to further degrade either reovirus core or purified CPV into smaller enzyme-active particles have succeeded only in eliminating polymerase activity, most probably by dissociating the enzyme-template complex. Whatever treatment, chemical or physical, is used to separate RNA from the viral proteins appears also to destroy biological activity. Furthermore, we have had to date no success in obtaining template-free viral proteins in any form which, when combined with exogenous viral RNA in the proper substrate-salt milieu (4, 13, 22), initiates transcription. Based on these facts, the gross structure of reovirus cores, CPV, and WTV are compared diagrammatically in the upper portion of Fig. 13. Because WTV is rather unstable to CsCl, we can only estimate the minimal structure of transcription which would be analogous to reovirus cores and purified CPV. We have assigned this role to fraction C from CsCl ($\rho = 1.425$ to 1.445 g/cm³) which, despite the harsh effects of high salt, retains transcriptase activity (Lewandowski, unpublished observation).

One obvious question to arise from this com-

FIG. 13. Top, Diagrammatic presentation of the structures of reovirus core, CPV, and WTV, representing what is known to date of the minimal structure of diplornavirus transcription. Bottom, Comparison of the molecular weights of select procaryotic and eucaryotic DNA-dependent RNA polymerase polypeptides with the viral polypeptides associated with the suggested minimal viral RNA-dependent RNA polymerase activities.

parison concerns which polypeptides among the total protein mass of the minimal enzyme-template complex are responsible for the actual process of transcription. Based primarily on the striking similarity between the polypeptide compositions of *E. coli* deoxyribonucleic acid (DNA)-dependent RNA polymerase and reovirus cores, it has been suggested that the reovirus cores are made up entirely of transcriptase (11). Similar comparisons can be made between the polypeptides of CPV and WTV, on one hand, and certain procaryotic and eucaryotic DNA-dependent RNA polymerases (5, 17, 34), on the other (lower portion of Fig. 13). The general pattern of these compositional similarities suggests to us that these viral and host enzyme activities may prove to be analogous, perhaps differing chiefly in their preference of double-stranded template, either DNA or RNA. Such a hypothesis raises the interesting possibility of an evolutionary relationship between dsRNA-containing viruses and those portions of procaryotic and eucaryotic genomes coding for polymerase activities. It must be cautioned, however, that in all cases, including reovirus, in lieu of a direct reconstitution assay as is available for the host polymerases, there is no direct evidence showing which of the viral polypeptides designated in Fig. 13 is responsible for the viral transcriptase activity. One attractive possibility is, for example, that each of the capsomeric-like structures seen on the surface of reovirus cores, CPV and WTV, in addition to representing a major entity of virus superstructure, is also a multicomponent, biologically ac-

tive aggregate equivalent to procaryotic and eucaryotic host polymerase.

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