JOURNAL OF VIROLOGY, Dec. 1977, p. 805-820
Vol. 24, No. 3

Printed in U.S.A.

Peptide Map Comparison of Infectious Pancreatic Necrosis Virus-Specific Polypeptides

PETER DOBOS* AND DAVID ROWE

Department of Microbiology, College of Biological Sciences, University of Guelph, Guelph, Ontario, Canada, N1G 2W1

Received for publication 28 June 1977

An investigation of virus-specific protein synthesis in infectious pancreatic necrosis virus (IPNV)-infected rainbow trout gonad cells was undertaken to find a relationship between the coding capacity of the virus genome (two segments of double-stranded RNA of 2.5×10^6 and 2.3×10^6 molecular weight) and the sizes and relative amounts of virus-specific proteins. Using polyacrylamide slab-gel electrophoresis and autoradiography, eight distinct virus-specific polypeptides were detected in infected, [^{35}S]methionine-labeled cells. These proteins may be grouped into three size classes on the basis of molecular weight: (i) large, α (90,000); (ii) medium, β_1 (59,000), β_2 (58,000), and β_3 (57,000); and (iii) small, γ_1 (29,000), γ_{1A} (28,000), γ_2 (27,000), and γ_3 (25,000). The combined molecular weight of these polypetides (373,000) is beyond the coding capacity of the virus genome. Purified IPNV contained polypeptides α, β_3, γ_1, and γ_{1A}. Pulse-chase experiments and tryptic peptide map comparisons revealed that only four of the eight intracellular proteins were primary gene products, namely, α, β_1, γ_1, and γ_2, with a combined molecular weight of 205,000. Of these primary gene products only the α polypeptide was found to be stable, whereas the other three underwent intracellular proteolytic cleavage during virus morphogenesis. Polypeptide β_1 was cleaved to generate β_2 and β_3; γ_1 was trimmed to produce γ_{1A}, and the only nonstructural primary gene product, γ_2, was found to be a precursor of γ_3. These results suggest that IPNV possesses a unique mechanism to synthesize three size classes of proteins using mRNA transcripts from two high-molecular-weight double-stranded RNA genome segments.

Infectious pancreatic necrosis virus (IPNV) is the causal agent of a contagious, high-mortality disease of young hatchery-reared salmonids. It replicates in a variety of fish cell lines at temperatures below 24°C (15). Virus multiplication takes place in the cytoplasm (15), resulting in a characteristic cytopathogenic effect (15). A single cycle of replication takes 16 to 22 h at 22°C, and a large proportion of the progeny virus remains cell associated (4, 11). Electron microscopic observations of purified virus revealed structures of naked icosahedrons, which were similar in size and shape to reovirus but lacked the characteristic double capsid of the latter (4, 11).

Recently, we have shown that the IPNV genome consists of two pieces of high-molecular-weight double-stranded (ds) RNA (2.5×10^6 and 2.3×10^6) sedimenting as an RNase-resistant 14S component in sucrose gradients (5). Upon denaturation the sedimentation constant increased to 24S, and the RNA became sensitive to RNase (5). Similar results were found more recently by Macdonald and Yamamoto (14) and by Underwood et al. (B. O. Underwood, C. J. Smale, F. Brown, and B. Hill, J. Gen. Virol., in press), indicating that IPNV belongs to the family of *Reoviridae*. However, since it contains only two segments of dsRNA, IPNV cannot be included in any of the established genera.

A recent comparison of the biophysical properties of IPNV to those of reovirus further indicated that the two viruses are completely different from one another (7). The molecular weight of IPNV was determined to be 55×10^6, using analytical ultracentrifugation and laser quasi-elastic light-scattering spectroscopy. Other biophysical and biochemical properties of IPNV also differed greatly from those of reovirus, for example: RNA content, 8.7%; sedimentation coefficient, 435S; hydrated diameter, 64 nm; and electron microscopic (anhydrous) diameter, 59 nm. The values for the sedimentation coefficient and the electron microscopic diameter of IPNV have been confirmed recently by Underwood et al. (in press).

The structural proteins of IPNV fall into three size classes, large, medium, and small, as has

been demonstrated in different laboratories (4, 6). This raises a paradox: how can a large-size-class dsRNA genome mediate the synthesis of three different size classes of proteins? To attempt to answer this question, we have analyzed recently the time course of virus-specific protein synthesis in cells infected by IPNV (6). Using acrylamide gel electrophoresis and autoradiography, we found that three size classes of virus-specific polypeptides were synthesized intracellularly, in the same relative proportion throughout the infectious cycle. There were two minor proteins of the large size class (α_1 and α_2) and several major proteins of medium (β) and small (γ) size classes. Purified virus contained the two large-, one of the medium-, and two of the small-size-class polypeptides. Pulse-chase experiments and techniques that prevented post-translational cleavage of proteins indicated that polypeptides of the β and γ families did not arise from the α proteins by proteolytic cleavage. In addition, the relative molar ratios of the three size classes of polypeptides indicated that the frequency of their synthesis was inversely related to their molecular weights. This type of protein synthesis might be expected to occur by the translation of three size classes of monocistronic mRNA's. Trimming of the larger β polypeptides could be demonstrated, since radioactivity was chased from them to the smallest of the β proteins, which is the most abundant protein found in purified virus. It was found that the combined molecular weights of the intracellular virus-specific polypeptides were beyond the coding capacity of the virion RNA, indicating that some of these proteins were not primary gene products.

The purpose of the study reported here was to further examine the relationship between the virus genome and virus-specific proteins by comparing the peptide maps of these proteins, to ascertain which of these polypeptides were primary gene products and to determine whether the combined molecular weights of these primary gene products were within the coding capacity of the virus genome.

The results show that: (i) polypeptides α_2, β_1, γ_1, and γ_2 are primary gene products and consequently their peptide maps are different from each other; (ii) α_1 is a dimer of β_3, the major viral capsid protein; (iii) β_1 is a precursor of β_2 and β_3, and their peptide maps are very similar; (iv) during virus maturation, the intracellular protein γ_1 (molecular weight, 29,000) is slowly "trimmed" to γ_{1A} (27,000), a hitherto undetected virus-specific protein, and these two polypeptides make up the small-size-class proteins of the virion ($v\gamma_1$ and $v\gamma_{1A}$) that have identical peptide maps; (v) the small nonstructural virus-specific protein γ_2 (26,000) is the precursor of the smallest intracellular virus-specific protein γ_3 (25,000), as shown by pulse-chase experiments and peptide mapping. The combined molecular weight of the primary gene products (α, β_1, γ_1, γ_2) is 205,000, well within the coding capacity of the virus genome (266,000 maximum).

The results indicate that IPNV has a unique mechanism for synthesizing three size classes of proteins, using mRNA transcripts from two high-molecular-weight segments of dsRNA.

MATERIALS AND METHODS

Cells. Rainbow trout gonad (RTG-2) cells were obtained from the American Type Culture Collection (ATCC). They were grown at 22°C as monolayers in plastic tissue culture vessels (Corning Plastics Inc.), using Eagle minimum essential medium (MEM) with Earle salts, supplemented with 10% (vol/vol) fetal calf serum and 40 μg of gentamycin per ml.

Virus. IPNV strain VR299 (ATCC) was propagated in RTG-2 cell monolayers at a multiplicity of infection of 0.01 PFU/cell. Infected cultures were incubated at 22°C unil extensive cytopathogenic effect was observed, usually 2 to 3 days. The cells were scraped into the tissue culture medium, the culture vessels were placed in crushed ice, and the cultures were sonically treated for 30 s with a Biosonik III sonicator at its maximum setting. This virus stock (10^8 to 5×10^8 PFU/ml) was dispensed in 1-ml samples and stored at −70°C.

Preparation of radioactively labeled purified virus. Cell monolayers (80 to 90% confluent) were infected with IPNV in a small volume of MEM at a multiplicity of infection of 0.1 to 1.0 for 1 h. The inoculum was replaced with methionine-deficient MEM (prepared by mixing 95 ml of methionine-free MEM with 5 ml of complete MEM) containing 5% dialyzed fetal calf serum and 6 μCi of [^{35}S]methionine per ml. After 24 h of incubation at 22°C, more [^{35}S]-methionine was added to the medium to increase its concentration to 12 μCi/ml, and the cultures were further incubated until extensive cytopathogenic effect was evident (2 to 3 days).

Virus purification was performed by using the method of Macdonald and Yamamoto (14). Briefly, the cells were scraped into the medium and sedimented by centrifugation at 5,000 rpm in the cold. The cell pellet was resuspended in 5 ml of TNE buffer (0.1 M Tris–0.1 M NaCl–1 mM EDTA, pH 7.3) and was extracted with 2 ml of Freon 113, using a Dounce homogenizer. To the culture medium containing virus, NaCl was added at a rate of 2.2 g/100 ml, and the medium was stirred in the cold with a magnetic stirrer. Polyethylene glycol 20,000 was slowly added to a concentration of 5% (wt/vol), and the mixture was stirred in the cold for 4 to 5 h. The polyethylene glycol-precipitated material was pelleted by centrifugation in the cold at 10,000 × *g* for 1 h. The supernatant was discarded, and the pellet was resuspended in the Freon-extracted virus sample. The preparation was layered over a stepwise gradient consisting of 3 ml of 40% (wt/vol) CsCl in TNE buffer, 3 ml of 30% CsCl, and 1 ml of 20% CsCl in a nitrocellulose ultracentrifuge

tube. Centrifugation was for 16 h at 39,000 rpm at 4°C, using the SW40 rotor in a Beckman L2-65B ultracentrifuge. The virus band was withdrawn by puncturing the side of the tube with a syringe, and the density was adjusted to 1.33 g/cm^3 in 5 ml of TNE buffer, using an Abbe refractometer (10). The virus was isopycnically banded at 100,000 × *g* for 16 h (SW50.1 rotor). The virus band, at the density level of 1.33 g/cm^3 (6), was again withdrawn by tube puncture, diluted to 3.5 ml with TNE buffer, layered onto a 2-ml column of 20% sucrose in TNE buffer, and centrifuged at 100,000 × *g* for 1 h (SW50 rotor) to pellet the virus. The supernatant was discarded, and the virus pellet was dissolved in 100 to 200 μl of electrophoresis sample buffer (ESB; 0.05 M Tris [pH 6.8], 1% [wt/vol] sodium dodecyl sulfate [SDS], 2 mM EDTA, 1% [vol/vol] 2-mercaptoethanol, 10% [vol/vol] glycerol, and a trace of bromophenol blue tracking dye). The preparation was heated for 2 min in a boiling-water bath and either analyzed immediately or stored in screwed-capped vials at −20°C until used. Since it was found that the mercaptoethanol tended to evaporate during storing, these samples, after thawing, received additional mercaptoethanol (up to 2%, vol/vol) before boiling and analysis in acrylamide gels.

Radioactive labeling of infected cells for polyacrylamide gel electrophoresis. Confluent RTG-2 cell monolayers in plastic tissue culture plates (6 cm in diameter) were infected with IPNV at 22°C at a multiplicity of infection of 10 to 20 PFU/cell. After 1 h was allowed for adsorption, the virus inoculum was replaced with growth medium and the cultures were incubated for an additional 2 to 3 h. At this time the medium was removed, the monolayers were rinsed three times with Earle balanced salt solution, and the uncovered plates were irradiated with UV light from a distance of 24 cm for 17 to 18 s, using a GE G8T5 germicidal lamp (44 ergs/s per mm^2 at 25 cm). The medium was replaced, and incubation was continued at 22°C as has been described previously (6). This manipulation brought about a great reduction in the rate of cell-specific protein synthesis and allowed the easy detection of (even the minor) virus-specific intracellular polypeptides. At various times postinfection, the growth medium was replaced with Earle balanced salt solution containing 5 μCi of [^{35}S]methionine per ml, and the cultures were incubated for various times, depending on the length of the "pulse." At the end of the labeling period, the cell layers were washed with fresh Earle balanced salt solution and the monolayers were dissolved in 0.5 ml of ESB. The preparation was forced through a small-gauge hypodermic needle to sheer the DNA and pretreated for gel electrophoresis as described above for purified virus.

In pulse-chase experiments the cells were washed three times, after the "pulse" with [^{35}S]methionine, and further incubated in complete MEM to which a 1,000-fold excess of unlabeled methionine was added. At the end of the "chase," the cultures were treated as above.

Polyacrylamide gel electrophoresis of proteins. Radioactively labeled proteins were analyzed in slab gels, using the discontinuous SDS-gel system of Laemmli (12). Gel slabs 10, 15, or 20 cm long and 0.1 cm thick were formed between glass plates. The general apparatus and methodology for electrophoresis followed the description of Studier (18); 10 and 12% resolving gels and 5% spacer gels were prepared from a stock solution in which the ratio of acrylamide to bisacrylamide was 30:0.8. Five to twenty microliters of labeled cell lysate or virus in ESB (containing 10,000 to 100,000 cpm) was loaded into the wells with a microsyringe (in preparative gels the spacer gel was formed without wells, using the "blank"). Electrophoresis was for 3 to 4 h at 120 V. After the run the slabs were stained in 0.1% (wt/vol) Coomassie brilliant blue in acetic acid-methanol-water (5:40:55), destained in the latter solvent, dried under vacuum (18), and placed in the dark on Kodak X-ray film (PR/R-2 Royal X-Omat) for autoradiography (2 to 10 days depending on the amount of radioactivity present in the samples).

For quantitative measurements, the bands containing the individual radioactive polypeptides were sliced out of the dry gel and dissolved in tightly capped scintillation vials with 0.1 ml of 30% H_2O_2 for 16 h at 60°C. The samples were cooled to −20°C for 1 h, 10 ml of Triton-toluene-based scintillation cocktail {to 2 liters of toluene containing 12 g of 2,5-diphenyloxazole (PPO) and 0.6 g of 1,4-bis[2]-(5-phenyloxazolyl)benzene (POPOP), 1 liter of anhydrous Triton X-100 was added} was added to each vial, and the radioactivity was measured in a liquid scintillation counter.

The molecular weights of IPNV-specific proteins were determined by comparing their electrophoretic mobilities in polyacrylamide gels with proteins of known molecular weight. The following marker proteins were used: reovirus proteins as characterized by Both et al. (2); encephalomyocarditis virus proteins as characterized by Dobos and Martin (8); phosphorylase A (molecular weight, 90,000); bovine serum albumin (67,000); gamma globulin H chain (50,000); ovalbumin (45,000); gamma globulin L chain (25,000); and tobacco mosaic virus protein (17,500).

Reduction and alkylation of proteins before acrylamide gel electrophoresis. Infected, labeled cell lysates were reduced and alkylated according to the method of Schlesinger and Schlesinger (17). Samples were reduced by dissolving infected cell monolayers in 0.1% SDS containing 0.28 M mercaptoethanol at pH 8.8, followed by incubation at 37°C for 1 h. Solid iodoacetamide was added to a final concentration of 0.3 M, and incubation was continued for an additional 1 h. The preparation was dialyzed for 12 h at room temperature, against 15% sucrose, 0.1% SDS, 1% mercaptoethanol, and 5 mM phosphate (pH 7.0), and analyzed immediately thereafter, without storing.

Analysis of tryptic peptides. Tryptic digestion of ^{35}S-labeled virus-specific polypeptides was carried out according to the method of Morrison and Lodish (16). Briefly, individual bands were cut from the stained, dried gels after exposure for autoradiography. The gel slices were chopped to 3-mm segments, swollen in a slight excess by volume of tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (50 μg/ml) in 0.2 M NH_4HCO_3 (pH 8.5), and incubated overnight at 37°C. Incubation was then continued for a further 6 h with a fresh batch of trypsin. Recovery of radioactivity from the gel slices was over 75%. The peptide digests were centrifuged at 5,000 rpm for 10

min to sediment small gel fragments, and the supernatants were lyophilized, washed with water, relyophilized, and finally resuspended in a small volume of electrophoresis buffer (pyridine-acetic acid-water, 1:10:100), pH 3.5. Insoluble material was removed by low-speed centrifugation. The separation of radioactive peptides was achieved by electrophoresis and chromatography on thin-layer sheets of silica gels (20 by 20 cm, 0.2 mm thick; Polygram SIL N-HR, Macherey-Nagel Co.). Samples of two digests to be compared were spotted onto a sheet about 3 cm from each other and 3 cm from the edge of the plate, on the two sides of a light pencil line, dividing the thin-layer chromatography plate into two halves composed of 20- by 10-cm squares. Loading of the digests was on the basis of radioactivity rather than volume due to the different specific activities of different samples. After allowing the samples to air dry, the sheets were lightly sprayed with electrophoresis buffer. A trace of phenol red was spotted in the corner of each sheet to serve as visual marker, and the peptides were subjected to electrophoresis at 300 V (10 mA) for 4 to 5 h or until the phenol red marker moved 8 cm.

After the run the sheets were dried and cut in half along the dividing pencil mark, and the two half-sheets (containing the two digests to be compared) were subjected to ascending chromatography together at room temperature in *n*-butanol–acetic acid–water (3:1:1) until the solvent front reached the top of the sheet. The dried plates were subjected to autoradiography for 10 to 30 days, using non-screen X-ray film.

Media and reagents. Fetal calf serum (virus and mycoplasma screened), MEM, and Earle balanced salt solution were purchased from Grand Island Biological Corp. The following materials were obtained from New England Nuclear Corp.: PPO, POPOP, Triton X-100, [^{35}S]methionine (400 to 600 Ci/mmol). Recrystallized acrylamide, bisacrylamide, and *N,N,N′,N″*-tetramethylethylenediamine were obtained from Bio-Rad Laboratories Ltd. SDS (specially pure) was obtained from British Drug Houses; and TPCK-trypsin was purchased from Sigma Chemical Co.

RESULTS

IPNV-specific polypeptides. IPNV infection does not decrease appreciably the rate of host cell protein synthesis. Therefore, to study the synthesis of virus-specific polypeptides in infected cells (especially that of the least abundant α proteins), it was necessary to develop a method whereby host cell protein synthesis could be preferentially suppressed without inhibiting virus replication. We have achieved this by UV irradiation of infected cell monolayers to destroy host mRNA, a technique used for the same purpose in T7 phage-infected bacterial cultures (18). The rationale for this technique was that the dsRNA of IPNV and the virion-associated transcriptase (3) should be more resistant to UV irradiation than host mRNA. Consequently, if infected cells were UV irradiated, host mRNA's would be destroyed, but the virion-associated transcriptase would continue to function, generating virus-specific mRNA's which, in turn, would be translated.

The effect of UV irradiation on protein synthesis in uninfected and infected cells is shown in Fig. 1. After UV treatment, few host cell-specific proteins became labeled (Fig. 1A and B), allowing the easy detection of virus-specific polypeptides. An autoradiogram of infected, UV-irradiated cells, pulse-labeled with [^{35}S]methionine for 4 h, is shown in Fig. 1C. The following virus-specific polypeptides can be distinguished: α, β_1, β_2, β_3, γ_1, γ_{1A}, γ_2 and γ_3. Purified virus contains only polypeptides α, β_3, γ_1, and γ_{1A} (Fig. 1D). The molecular weights of these polypeptides are listed in Table 1.

Polypeptide α. Our earlier study indicated the presence of two high-molecular-weight polypeptides in the virion, α_1 and α_2 (molecular weights, 100,000 and 90,000, respectively) (Fig. 1F and 2C; 6). These polypeptides were also observed in gel profiles of isotopically labeled infected cell lysates that had been stored in ESB at −20°C. More recently, however, we have found that freshly prepared material (either virus or infected-cell lysate) contained only one α protein (Fig. 1C and D, 4, and 6; 7), which did not separate into two components even in 30-cm-long acrylamide gels (Fig. 6). It appeared, therefore, that the additional α protein was an artefact, arising either by proteolytic degradation of α_1 or through dimerization of one of the β-polypeptides. Since the addition of phenylmethylsulfonyl fluoride, a protease inhibitor, did not eliminate any one of the two α proteins (data not shown), the second alternative, namely, dimerization of one of the β polypeptides, seemed more likely. To investigate this possibility, labeled infected-cell lysates were reduced and alkylated before acrylamide gel electrophoresis. This treatment led to the disappearance of the α_1 protein, whereas α_2 remained unchanged (Fig. 2B). When purified top component or virus was dissolved in ESB without mercaptoethanol, β_3 protein formed dimers (Fig. 1E and F). Since top component contains only β_3 protein (Fig. 6C; 7) oligomers can only result from this polypeptide. Furthermore, when infected, labeled cell lysates were dissolved in ESB from which mercaptoethanol was omitted, dimers, trimers, and tetramers of β_3 formed (Fig. 2A). Dimerization of β_3 also took place when the sample was heated in ESB and stored at −20°C for a few weeks before analysis (Fig. 2C). Apparently, mercaptoethanol slowly evaporated during storage, which allowed dimer formation of β_3. The molecular weights of these oligomers of β_3 fell on a straight line (Fig. 3) when plotted

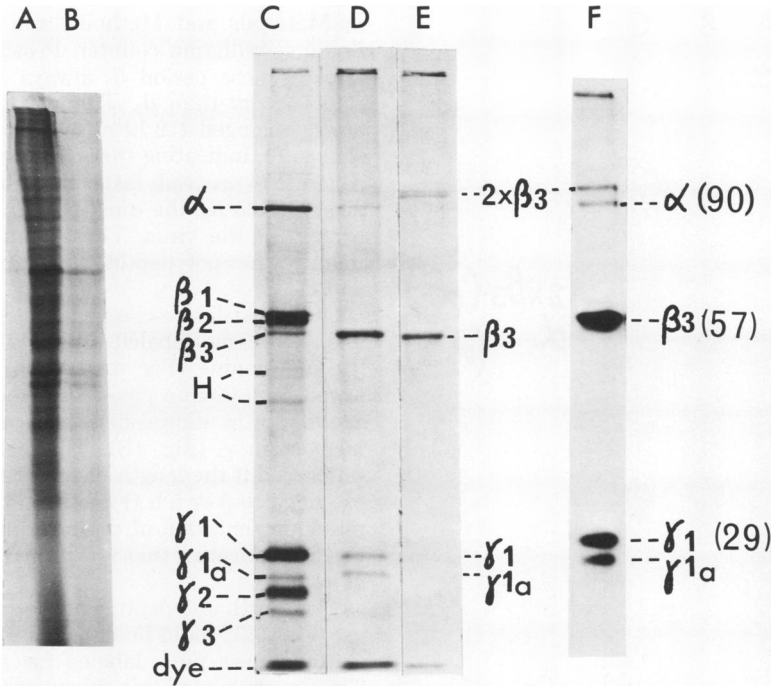


FIG. 1. *Autoradiograms of labeled polypeptides of uninfected, infected, and UV-irradiated cells, purified virus, and top component analyzed on 10% polyacrylamide Laemmli-type slab gels. (A) Polypeptide profile of uninfected RTG-2 cells labeled with [^{35}S]methionine for 4 h; (B) uninfected cells similarly labeled after UV irradiation as described in the text; (C) infected cells UV irradiated at 2 h postinfection and labeled for 4 h at 6 h after infection; (D) labeled purified virus; (E) labeled top component dissolved before electrophoresis in hot SDS without mercaptoethanol; (F) purified virus dissolved in hot SDS without mercaptoethanol. Material in (C), (D), and (E) were analyzed together in the same slab gel; therefore, the electrophoretic mobilities of the different polypeptides are directly comparable. H denotes host-cell-specific proteins. Dimer of β_3 is represented by the symbol 2 × β_3. The numbers in parentheses denote the molecular weights of polypeptides α, β_3, and γ_1 (see Table 1).*

TABLE 1. *Molecular weights of IPNV-specific polypeptides*

Designation	Mol wt[a]	
	Infected cell	Virion
α	90,000	90,000
β_1	59,000	
β_2	58,000	
β_3	57,000	57,000
γ_1	29,000	29,000
γ_{1A}	28,000	28,000
γ_2	27,000	
γ_3	25,000	

[a] The molecular weights of virus-specific polypeptides were determined by comparing their electrophoretic mobilities to marker proteins of known molecular weight, as described in Materials and Methods.

on a semilogarithmic scale, further indicating that β_3 tends to polymerize in the absence of reducing agents.

These results indicate that polypeptide α_1 is not a primary gene product, as previously thought (6). Consequently, there is only one large-size-class, virus-specific polypeptide, namely, α (by previous nomenclature α_2 [6]). The combined molecular weight of the remaining virus-specific polypeptides (α, β_1, β_2, β_3, γ_1, γ_{1A}, γ_2, and γ_3) is 373,000, still more than the theoretical maximum of 266,000 for which the virus genome can code. Therefore, it was necessary to further investigate the possible precursor-product relationship(s) among these polypeptides, using pulse-chase experiments and peptide mapping.

Pulse-chase experiments. When infected, UV-irradiated cells were pulse-labeled with [^{35}S]methionine for 60 min at 5 h after infection, only β_1 of the β polypeptide family became labeled (Fig. 4A). When the 60-min pulse was followed by a 1-h chase with medium containing an excess of unlabeled methionine, radioactivity was chased from β_1 into β_2 and β_3 (Fig. 4B). The gradual disappearance of radioactivity from β_1 and its appearance in β_2 and β_3 was easily observable if the length of the chase period was

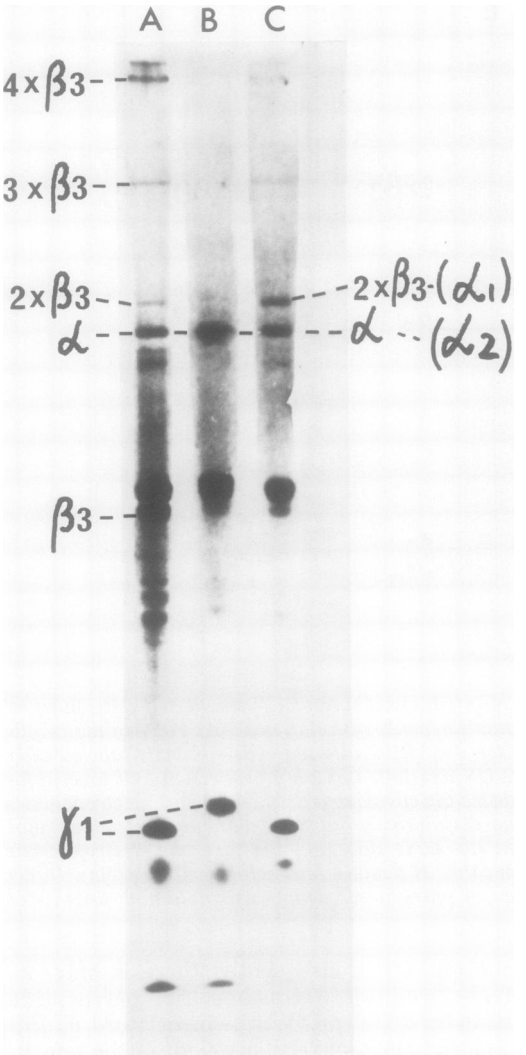


FIG. 2. *Autoradiogram of labeled polypeptides in UV-irradiated, virus-infected cells analyzed on a 10% acrylamide slab gel. Infected, UV-irradiated cells were labeled with [^{35}S]methionine for 4 h at 6 h after infection. (A) Infected labeled cells dissolved in ESB that contained no mercaptoethanol; (B) similarly labeled cells dissolved in complete ESB (with mercaptoethanol) followed by reduction and alkylation of polypeptides as described in the text; (C) similarly labeled cells dissolved in compete ESB and stored at −20°C for 2 weeks before electrophoresis. Dimers, trimers, and tetramers of β_3 are signified by 2, 3, and 4 × β_3. Symbols in parentheses indicate previous designation of α polypeptide(s) (6).*

increased (3 h, Fig. 4D, or 5 h, Fig. 4E). A quantitative presentation of these results is shown in Fig. 5A. To construct the graph, the various virus-specific bands were sliced out of the dried gel slab, dissolved in H_2O_2 as described in Materials and Methods, and counted in a liquid scintillation counter. It can be seen that after a chase period β_3 always contained less radioactivity than β_2. This was observed even after prolonged (12 h) chase periods (data not shown; 6), indicating that the trimming of β_1 to generate β_2 proceeds faster than the process that gives rise to β_3, the most prominent structural protein of the virus. These results seemed to indicate that polypeptide β_1 is a precursor of β_2 and β_3.

Of the small-size-class γ polypeptides, only γ_1 and γ_2 became labeled after a 1-h pulse with [^{35}S]methionine (Fig. 4A). When this was followed by a 60-min chase the amount of radioactivity in γ_2 diminished, with a concomitant increase in γ_3 (Fig. 4B). This observation was reinforced if the length of the chase period was extended to 3 or 5 h (Fig. 4D and E). A quantitative presentation of these results is shown in Fig. 5B, indicating that γ_2 is probably a precursor of γ_3.

The fourth polypeptide of the small size class, γ_{1A} (Fig. 1C), could be detected only if infected cultures were pulse-labeled for 4 h or longer. The emergence of this minor protein was very slow compared with that of the other virus-specific polypeptides. Therefore, it was not feasible to demonstrate its relationship to other proteins with pulse-chase experiments, and its identity was established by tryptic peptide mapping (see below).

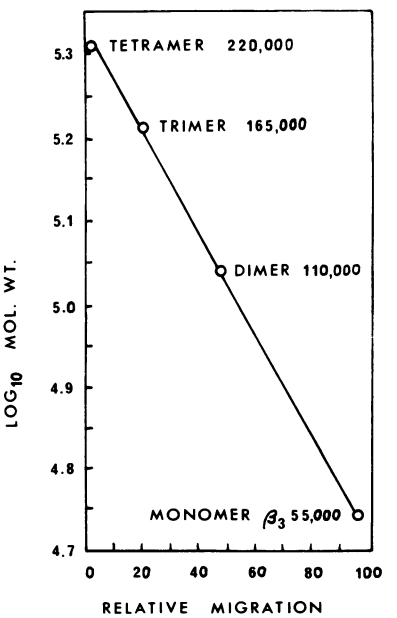


FIG. 3. *Semilogarithmic plot of dimers, trimers, and tetramers of polypeptide β_3 shown in Fig. 2A.*

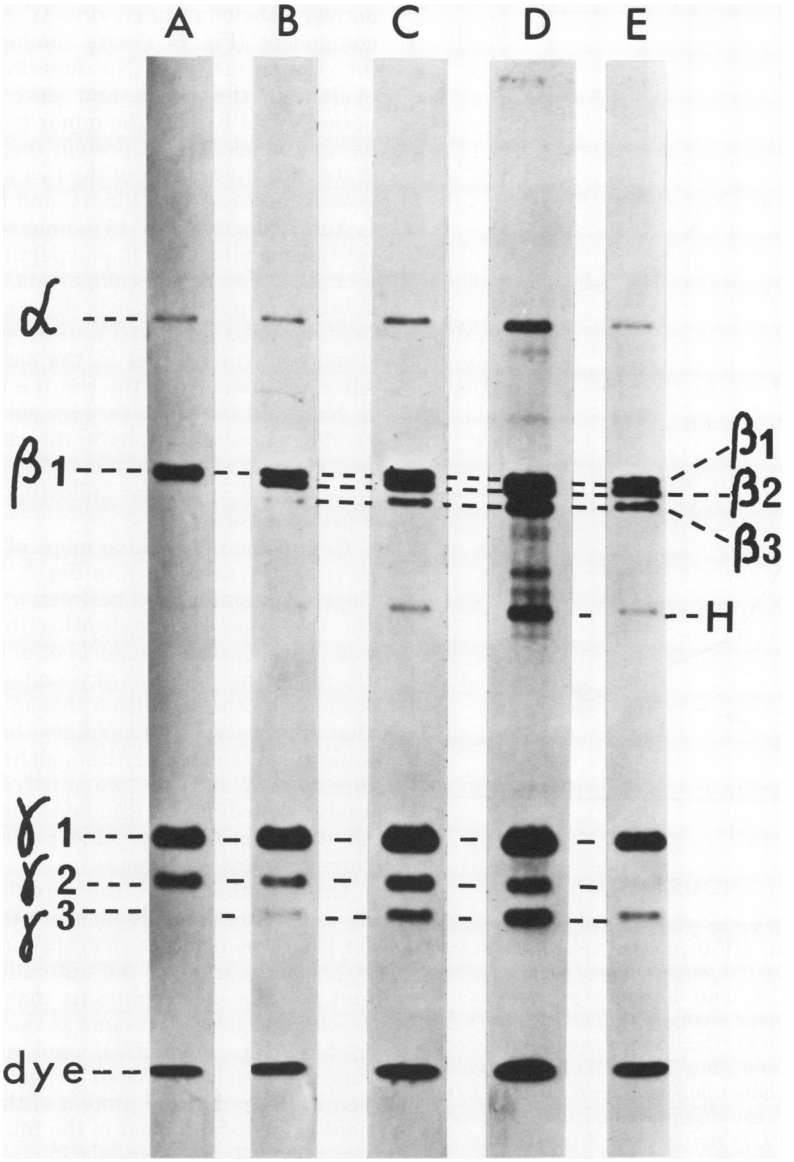


FIG. 4. *Autoradiograms of labeled intracellular virus-specific polypeptides analyzed on 10% slab gels. (A) Polypeptide profile of infected, UV-irradiated RTG-2 cells labeled with [^{35}S]methionine for 1 h at 5 h after infection; (B) infected, UV-irradiated cells labeled for 1 h at 5 h after infection followed by 60-min chase, using an excess of unlabeled methionine; (C) infected, UV-irradiated cells labeled for 3 h at 5 h after infection; (D) infected, UV-irradiated cells labeled for 2 h at 5 h after infection, followed by a 3-h chase period; (E) infected, UV-irradiated cells labeled for 2 h at 5 h after infection, followed by a 5-h chase with excess of unlabeled methionine. H denotes host cell protein.*

Tryptic peptide mapping of virus-specific polypeptides. Although the pulse-chase experiments indicated that β_1 was a precursor of β_2 and β_3, and that γ_2 was the precursor of γ_3, a tryptic peptide map comparison of the different polypeptides was clearly desirable to ascertain that this was indeed the case, and to demonstrate that the amino acid sequences of the α, β_1, γ_1, and γ_2 polypeptides were completely different from each other.

To this end, infected, UV-irradiated cultures were labeled with [^{35}S]-methionine from 6 to 10 h postinfection. Under these conditions all virus-specific proteins became labeled (Fig. 1C). At

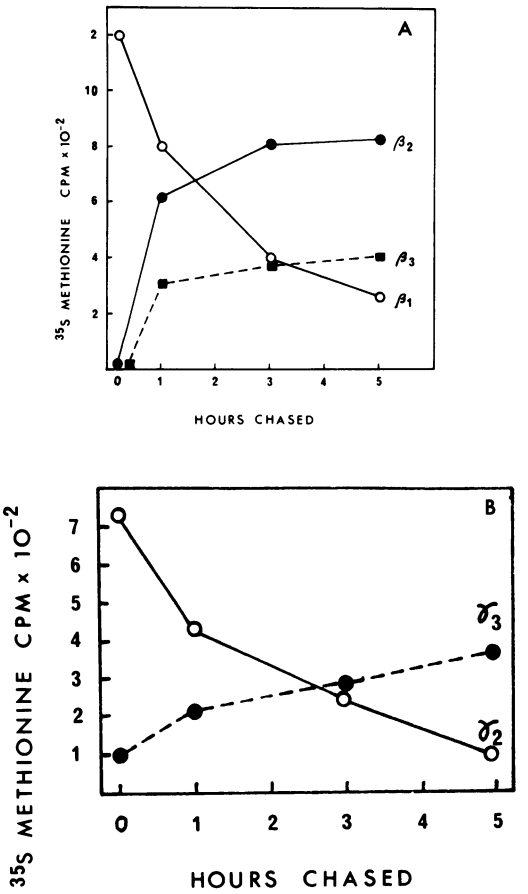


FIG. 5. *(A) Effect of chase on polypeptides of the β family. Infected, UV-irradiated cells were pulse-labeled for 1 h with [^{35}S]methionine at 5 h postinfection, followed by 1-, 3-, and 5-h chase periods using an excess of unlabeled methionine. The cells were lysed and subjected to acrylamide slab-gel electrophoresis followed by autoradiography. With the autoradiograms as templates, polypeptides β_1, β_2, and β_3 were sliced out from the dried gel slab and dissolved in 30% H_2O_2 at 60°C in tightly capped scintillation vials, and the radioactivity was counted in a liquid scintillation counter. (B) Effect of chase on polypeptides γ_2 and γ_3. The autoradiogram used to construct the graph in (A) was also utilized to slice out the γ_2 and γ_3 polypeptides. These were processed, and the radioactivity was measured as for those of the β family described in (A).*

the end of the labeling period the cells were dissolved in ESB, heated in a boiling-water bath for 2 min, and analyzed in 30-cm-long preparative slab gels followed by autoradiography (Fig. 6A). With the autoradiograms as templates, the virus-specific polypeptides were sliced out from the dried gels and subjected to tryptic digestion as described in Materials and Methods. Radioactively labeled purified virus (Fig. 6B) and top component (Fig. 6C) were similarly processed for peptide mapping. No dimerization of β_3 is evident in these gels, and since the labeling period was 4 h, even the minor polypeptide γ_{1A} became labeled. This protein seemed to comigrate with the smaller of the two γ polypeptides present in the virion (Fig. 1C and D, 6B).

Autoradiograms of two-dimensional tryptic peptide maps of α and β_1 polypeptides are shown in Fig. 7. For direct comparison (here and in subsequent peptide maps), digests of two polypeptides to be compared were subjected to electrophoresis in parallel in the same thin-layer silica gel plate. After the run the plate was cut in half, and both halves were subjected to ascending chromatography in the same tank. The peptide map of α protein is completely different from that of β_1, further indicating that α is not a precursor of β_1.

Comparison of peptide maps of polypeptides α, γ_1, and γ_2 are shown in Fig. 8. There are no fingerprint similarities between α and either of the two γ proteins, indicating that neither γ_1 nor γ_2 are cleavage products of α. Furthermore, the fingerprint patterns of γ_1 and γ_2 are different from each other and from that of β_1, indicating that these two polypeptides have different amino acid sequences. Although here the tryptic digests of β and the two γ polypeptides were analyzed on different thin-layer plates, the overall dissimilarity of peptide patterns of these three proteins is obvious.

On the other hand, the peptide maps of β_1, β_2, and β_3 are virtually identical (Fig. 9), except for the three peptides that appear at the upper right-hand corner of the autoradiograms of β_2 and β_3. The electrophoretic mobility of β_3 in acrylamide gels is the same as that of the virion protein (Fig. 1C and D), and most likely β_3 represents cell-associated progeny virus. The peptide map of the β protein of the top component is identical to that of the full virion and to the intracellular virus-specific polypeptide β_3 (data not shown). Thus, the comparison of peptide maps of the three β polypeptides supports the evidence obtained by pulse-chase experiments that during virus morphogenesis β_1 is trimmed to β_2 and β_3, the latter being the final size of this protein as it appears in mature progeny virus.

The peptide map of γ_2 is different from that of γ_1 (Fig. 8) but very similar to that of γ_3 (Fig. 10A), supporting the results of pulse-chase experiments (Fig. 5) that γ_2 is a precursor of γ_3.

The tryptic peptide patterns of γ_1 and γ_{1A} from infected cells are identical (Fig. 10B). Intracellular γ_{1A} only becomes labeled when long pulse periods are used (4 h or more). Thus the

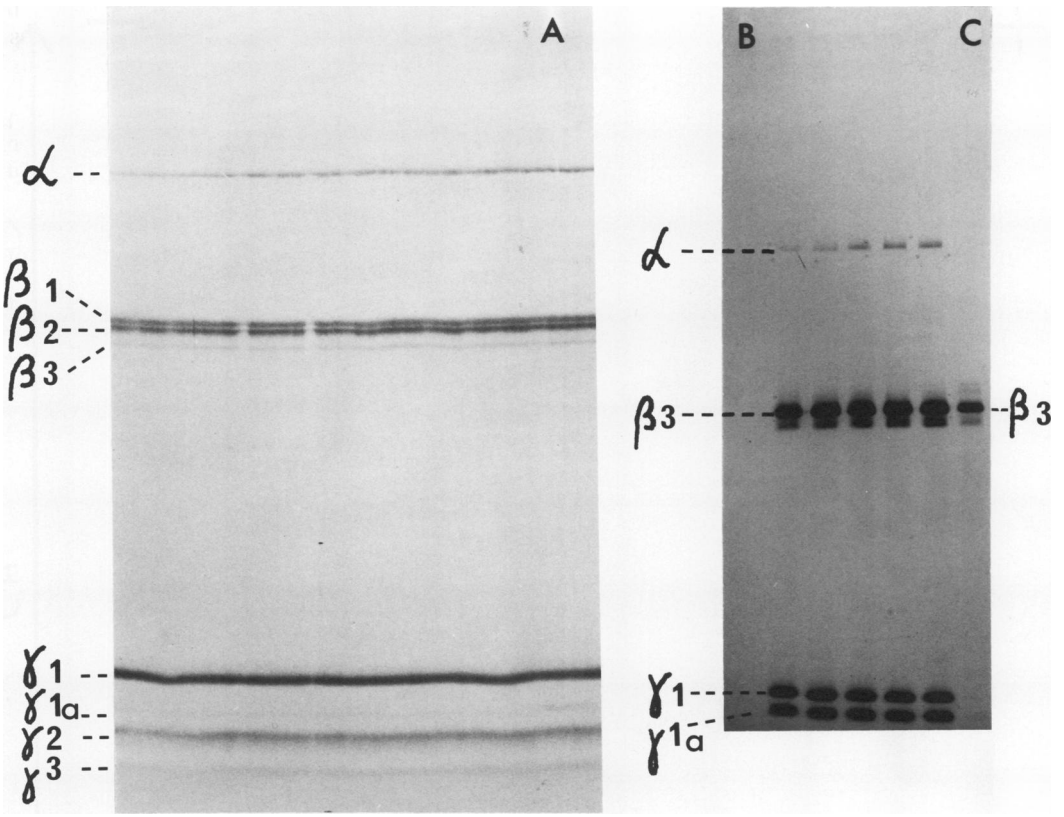


FIG. 6. *Preparative acrylamide gel electrophoresis of labeled virus-specific proteins. (A) Infected, UV-irradiated cells were labeled with [^{35}S]methionine for 4 h from 6 to 10 h after infection. The cells were lysed by heating in ESB followed by analysis in 30-cm-long 10% acrylamide slab gels. In this case, instead of the Lucite comb, a Plexiglas blank was used to form a single, long sample slot in the spacer gel. After electrophoresis the gels were stained with Coomassie brilliant blue, destained, dried, and placed on X-ray film for autoradiography as described in the text. The autoradiogram was used as a template to slice out the labeled virus-specific bands from the dried gel for trypsin digestion. (B) Preparative acrylamide gel electrophoresis of purified virus and (C) top component on 30-cm-long slab gels. The analysis and subsequent processing of the labeled material were performed as described for (A).*

cleavage of γ_1 to γ_{1A} is a relatively slow process and is most likely associated with virus maturation. In acrylamide gels the smallest γ polypeptide from purified virus preparations comigrates with γ_{1A} from infected cells (Fig. 2C and D) and not with γ_2 as previously thought (6), suggesting that both the intracellular and virion γ_{1A} polypeptides are cleavage products of γ_1.

Peptide map comparison of these polypeptides proves this point, since the fingerprint patterns of virions γ_1 and γ_{1A} are identical to each other and to those of the intracellular γ_1 and γ_{1A} polypeptides (Fig. 10B).

DISCUSSION

The present work identified eight IPNV-specific polypeptides in infected cells. These fall into three size classes: large (α), medium (β_1, β_2, and β_3), and small (γ_1, γ_{1A}, γ_2, and γ_3). The combined molecular weight of these proteins is 373,000. In a previous communication (6) we described an additional polypeptide of the large size class, designated α_1 (molecular weight, 100,000). As we show in this communication, polypeptide α_1 is not a primary gene product but represents a dimer of β_3 (Fig. 1, 2, and 3). Reduced and alkylated infected-cell lysates did not show this protein when analyzed in polyacrylamide gels (Fig. 2C), nor was it detected in freshly prepared lysates (Fig. 1, 4, and 6). On the other hand, when infected cells were dissolved by heating in ESB from which the reducing agent mercaptoethanol was omitted, not only dimers but higher-molecular-weight oligomers

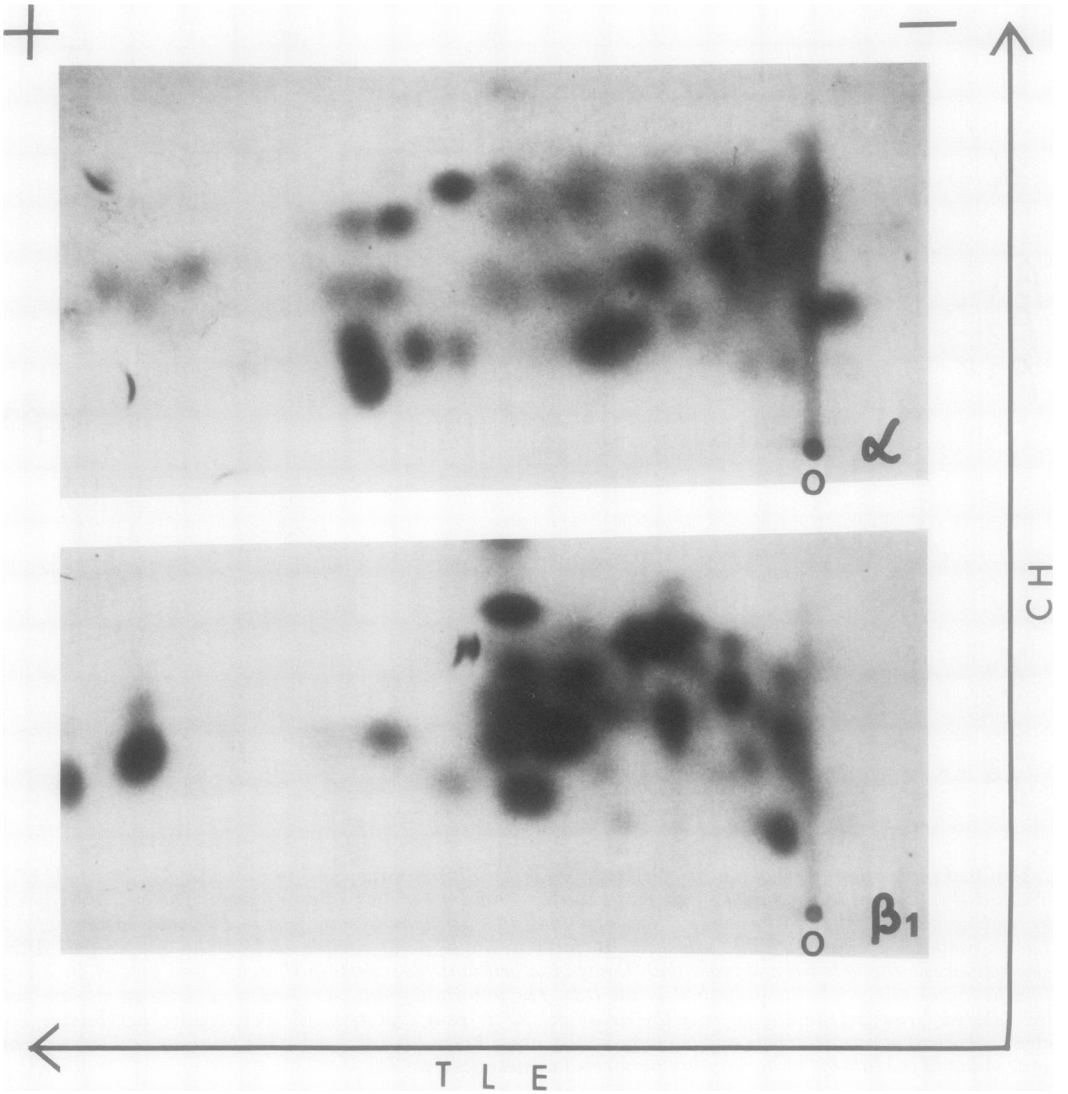


FIG. 7. *Fingerprint analysis of tryptic digests of intracellular virus-specific polypeptides. After acrylamide gel electrophoresis of infected, [^{35}S]methionine-labeled cells (Fig. 6), individual polypeptide bands were eluted by digestion with trypsin as described in the text. Samples were applied to bottom right corners* (○) *(as shown here and in subsequent figures) of thin-layer silica gel plates. Electrophoresis was at pH 3.5 with the anode to the left. Phenol red was spotted onto the left-hand corner of the plate, to serve as visual marker, and the digests were subjected to electrophoresis at 300 to 350 V until the phenol red spot moved 7 cm toward the cathode. Chromatography was toward the top of the sheet. For direct comparison, two samples were subjected to electrophoresis in parallel on the same thin-layer plate, which was cut in half for subsequent chromatography. Loads in counts per minute were, in (A), 6,000 for α and 5,000 for β_1. Autoradiography was for 5 weeks. (TLE and CH signify thin-layer electrophoresis and chromatography, respectively.)*

(trimers and tetramers) of polypeptide β_3 also formed (Fig. 2A and 3). One cannot conclude, purely on the basis of the molecular weight determination of these oligomers, that they are multiples of β_3. By this criterion alone they could equally be oligomers of β_1 or β_2, since the molecular weights of the β polypeptides differ but little from one another. We believe our conclusion that these oligomers represent β_3 (and not β_1 or β_2) is justified by the data presented in Fig. 1D, E, and F. The gel profiles of the two purified virus preparations differ in that an additional high-molecular-weight component is present ($2 \times \beta_3$ in Fig. 1F) which migrates slower

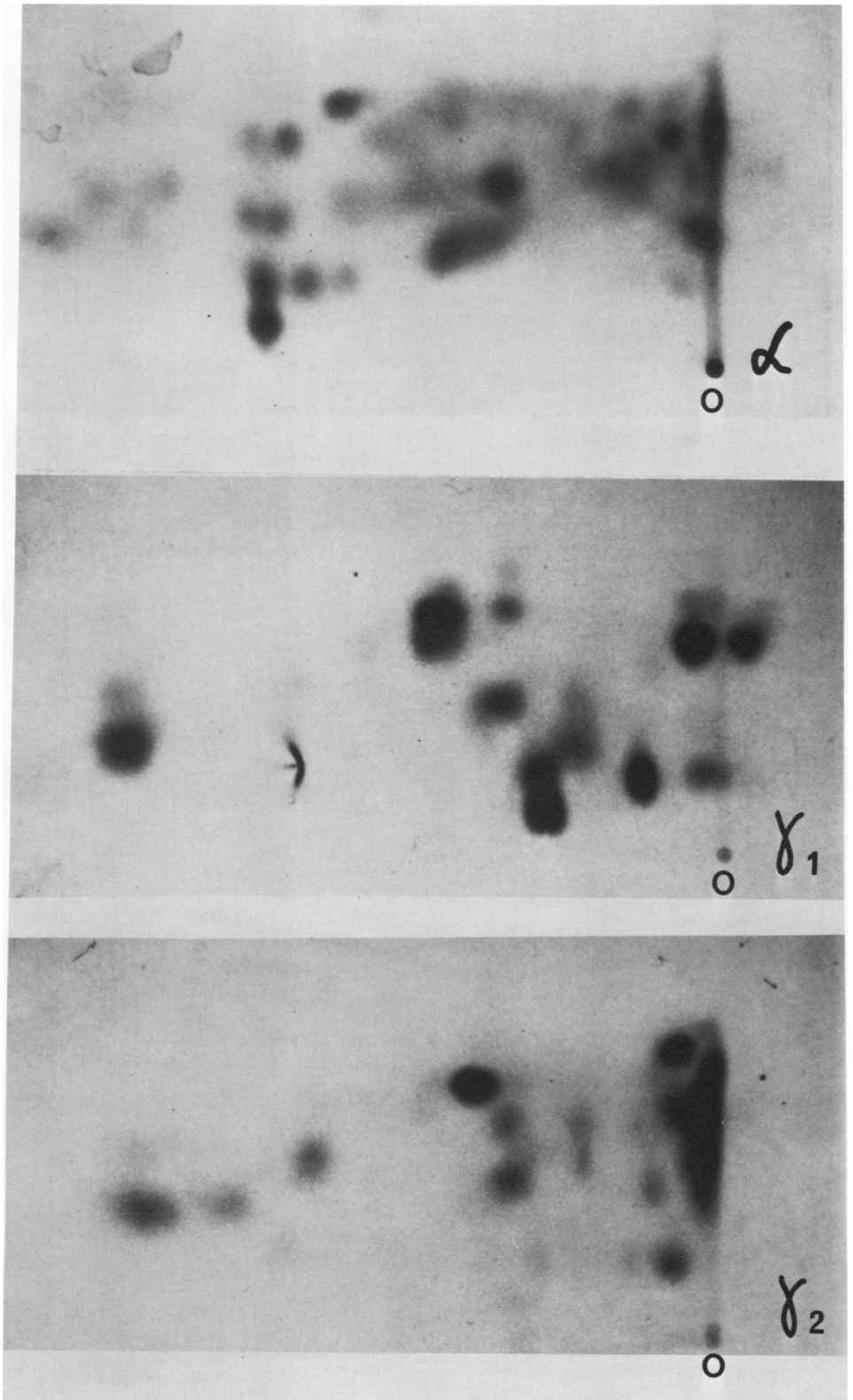


FIG. 8. *Tryptic peptide maps of [^{35}S]methionine-labeled, virus-specific polypeptides α_1, γ_1, and γ_2. Conditions for trypsin digestion, electrophoresis, and chromatography were as described in the legend to Fig. 7. Loads in counts per minute were 7,000 for each polypeptide.*

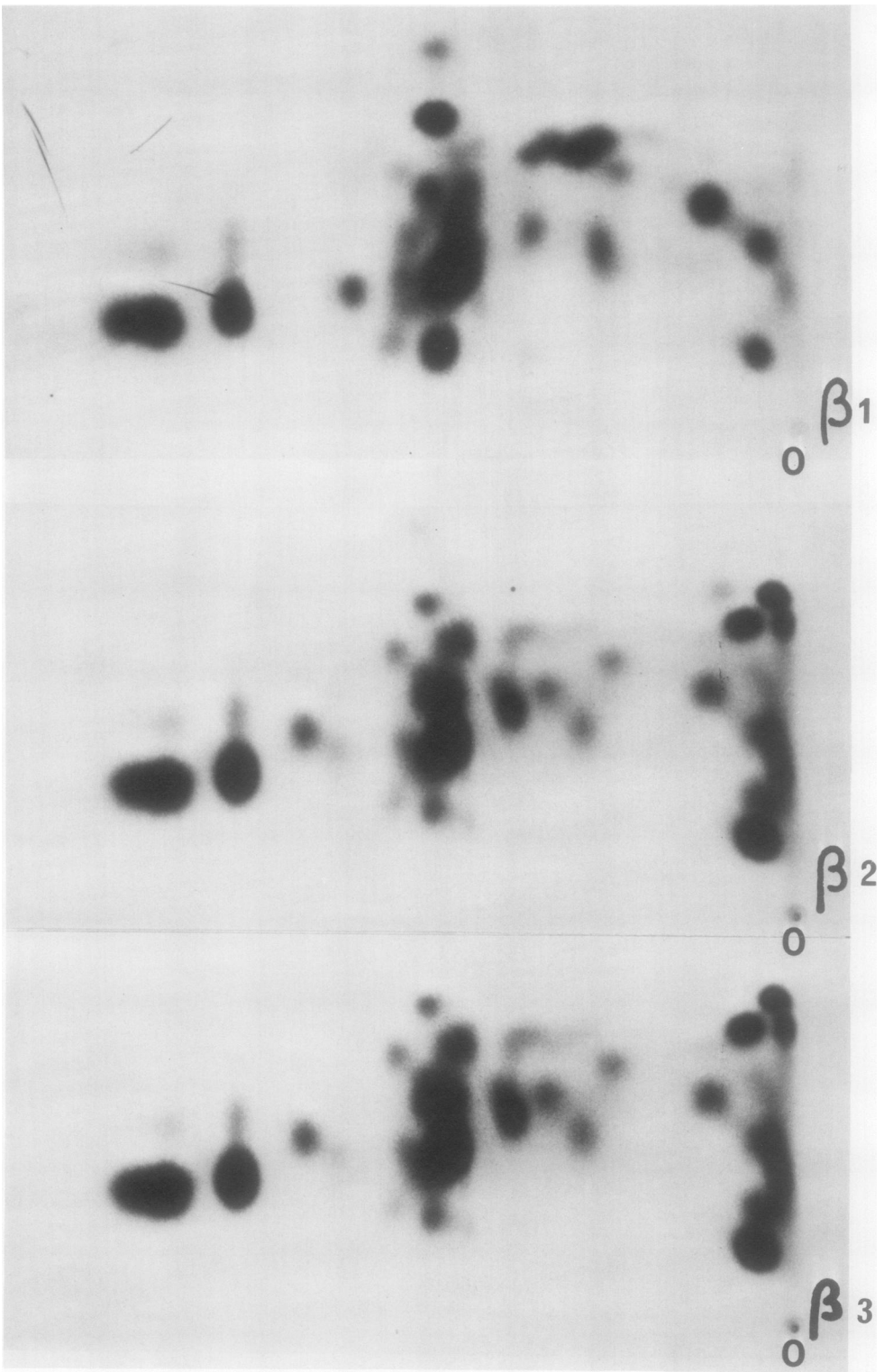


FIG. 9. *Tryptic peptide maps of [^{35}S]methione-labeled, virus-specific polypeptides β_1, β_2, and β_3. Conditions for trypsin digestion, electrophoresis, and chromatography were as described in the legend to Fig. 7. Loads in counts per minute were 7,000 for each polypeptide.*

than α. In this case purified virus was dissolved by heating in ESB without mercaptoethanol before gel electrophoresis. Since the virion does not contain β_1 or β_2 polypeptides, only the dimerization of β_3 can explain the appearance of this high-molecular-weight protein (designated α_1 in a previous communication [6] and indicated in parentheses in Fig. 2). This conclusion is reinforced by the data presented in Fig. 1E that shows the gel profile of top component, dissolved for electrophoresis without mercaptoethanol. Since the top component contains only β_3 (Fig. 6; 7), the high-molecular-weight minor component can only arise from the dimerization of this polypeptide.

It is interesting to note that oligomers of β_1 and β_2 were not formed, at least not in detectable quantities, when infected cells were dissolved in ESB without mercaptoethanol before electrophoresis. The polypeptides designated as $2 \times \beta_3$, $3 \times \beta_3$, and $4 \times \beta_3$ in Fig. 2A each appear as a single band, and not as a doublet or a triplet, which would be the case if each polypeptide of the β family were capable of forming oligomers.

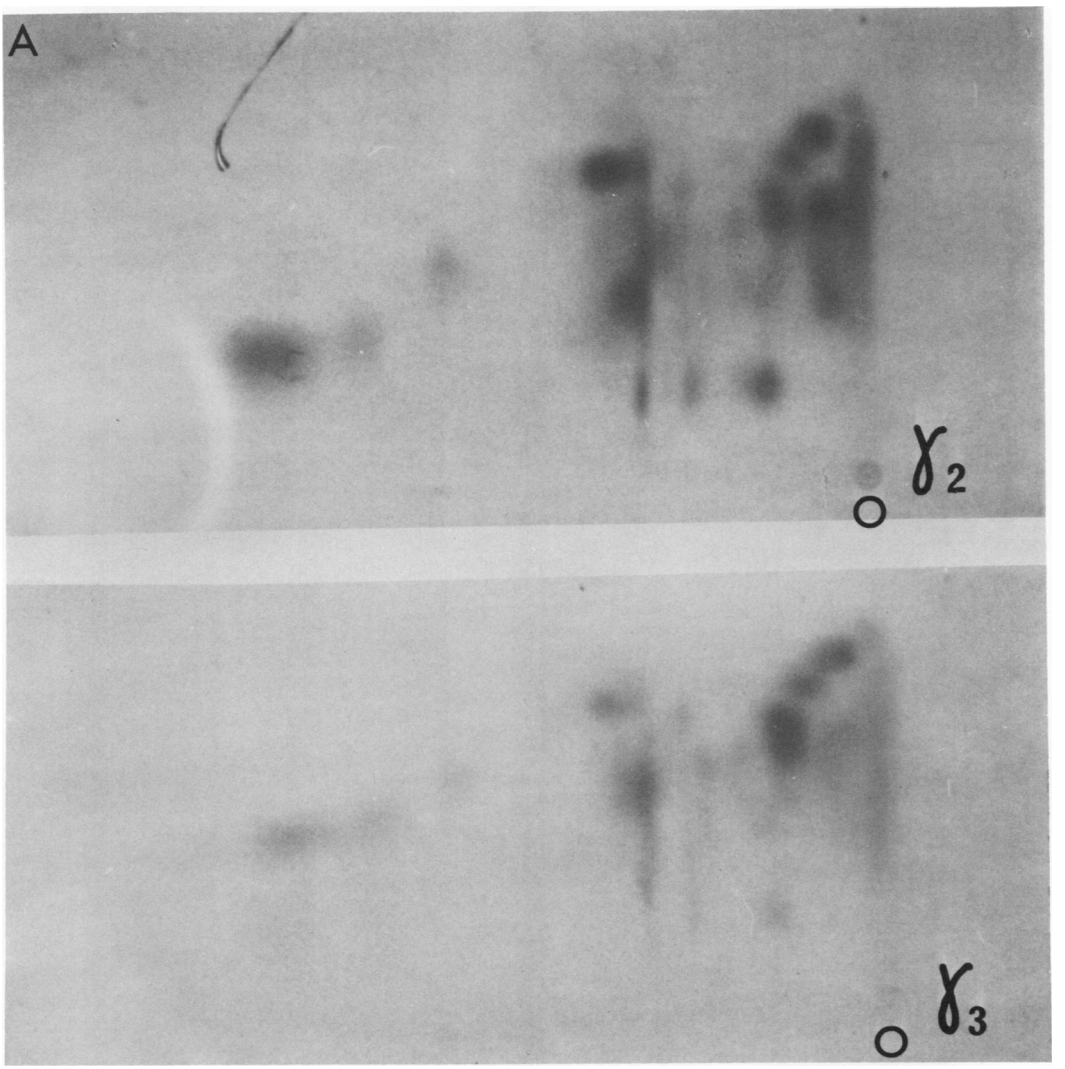
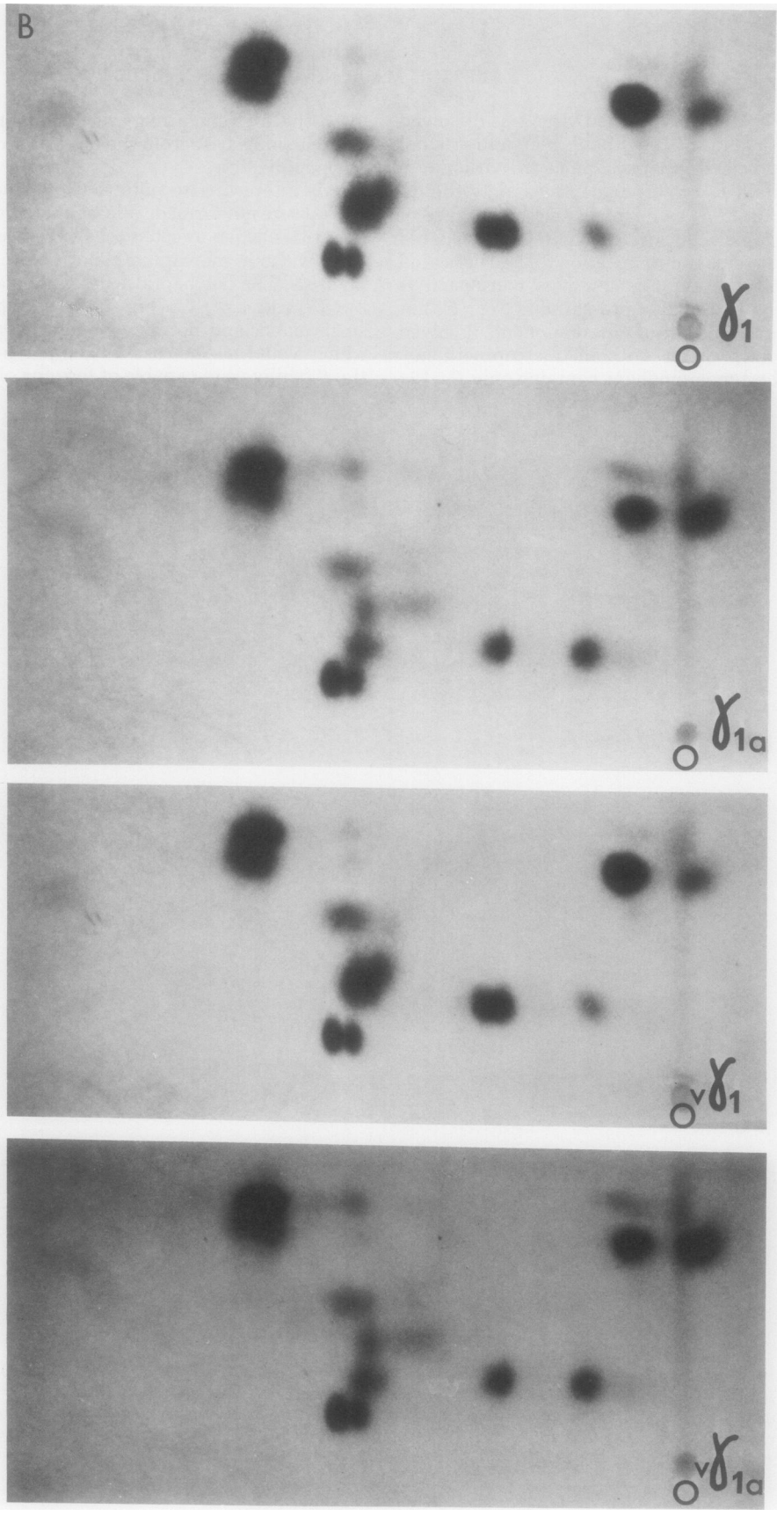


FIG. 10. *Tryptic peptide maps of [^{35}S]methionine-labeled, virus-specific polypeptides. (A) Fingerprints of γ_2 and γ_3; loads in counts per minute were 2,500 for each polypeptide. (B) Fingerprints of intracellular γ_1 and γ_{1a} as well as those from purified virus $V\gamma_1$ and $V\gamma_{1a}$. Loads in counts per minute were 6,000 for each polypeptide. In this case electrophoresis took place for 10 h instead of the usual 6 to 7 h, or until the phenol red marker migrated 10 cm (instead of the usual 7 cm).*


B
γ1
γ1a
Vγ1
Vγ1a

The combined molecular weight of the eight virus-specific proteins (Table 1) (373,000) is beyond the coding capacity of the virus genome (a maximum of 266,000-molecular weight protein), indicating that some of these polypeptides are not primary gene products. Pulse-chase experiments revealed that polypeptide β_1 is a precursor of β_2 and β_3 (Fig. 4 and 5). It is not known whether β_1 is trimmed to β_2, which in turn is trimmed to β_3, or that the β_1 polypeptide population is cleaved by two different proteolytic enzymes, one generating β_2 and the other generating β_3. Since the peptide maps of β_2 and β_3 are virtually identical (Fig. 9), the second alternative is possible only if the terminal regions of β_1 (which are cleaved off) contain no methionine. In this case a change in molecular weight could occur without a change in the peptide map. Alternatively, β_2 may not be trimmed at all but may be phosphorylated or otherwise modified, thus giving rise to β_3. Further research is needed to clarify this point.

The relationship among the different γ polypeptides was also investigated, using pulse-chase experiments and peptide mapping. The data presented in Fig. 4 and 5 indicate that γ_1 and γ_2 are different polypeptides and that γ_2 is a precursor of γ_3, since radioactivity could be chased from γ_2 to γ_3. Peptide map comparisons of γ_1 and γ_2 (Fig. 8) and γ_2 and γ_3 (Fig. 10A) justify this conclusion.

Peptide map comparison of α with β_1 (Fig. 7) indicates that they are different polypeptides. The dissimilar fingerprints of α, γ_1, and γ_2 (Fig. 8) also indicate that these proteins are unrelated to each other. These results show that neither β_1 nor γ_1 nor γ_2 is a cleavage product of α. The amino acid sequences of β_1, γ_1, and γ_2 are also different from each other, as borne out by their peptide map.

The fingerprints of γ_1 and γ_{1A} are identical to each other and to those of the two smallest virions polypeptides ($v\gamma_1$ and $v\gamma_{1A}$) (Fig. 10B). Polypeptide γ_{1A} can only be detected by using long labeling periods (4 h or longer; Fig. 1C and 6A), indicating that this protein is produced relatively slowly by the trimming of γ_1 and its production is probably associated with progeny virus maturation. Again, as for β_3 above, it is possible that γ_{1A} is a phosphorylated or otherwise modified form of γ_1 rather than a cleavage product. The data presented in Fig. 10B indicate that the smaller structural protein of the virus ($v\gamma_{1A}$) is identical to intracellular γ_{1A} and not with γ_2 as previously thought (6).

These results show that only four of the eight intracellular virus-specific polypeptides are primary gene products, namely, α, β_1, γ_1, and γ_2. The least abundant large-size-class α polypeptide is not cleaved, whereas the other middle- and small-size-class primary gene products all undergo processing during virus morphogenesis. β_1 is the precursor of β_2 and, either directly or via β_2, of β_3, which is the major structural component of the virion. γ_1 is slowly modified to produce γ_{1A} in infected cells even though both polypeptides appear as structural proteins in the purified virus. Polypeptide γ_2 and its cleavage product γ_3 are nonstructural proteins.

The combined molecular weight of the four primary gene products (α, β_1, γ_1, and γ_2) is approximately 205,000, well within the coding capacity of the virus genome.

The relationship between the virus-specific proteins and the virus genome still remains to be determined and is presently under investigation. It is interesting to speculate as to how two large dsRNA genome segments could mediate the production of three different size classes of polypeptides. For example, polypeptide α could be the product of one of the genome segments via the translation of a large-genome-length mRNA. Indeed, a 24S virus-specific mRNA was reported recently in IPNV-infected cells (1). This would leave the second genome segment to code for polypeptides β_1, γ_1, and γ_2. This would have to occur by a mechanism different from that found in reovirus-infected cells (19). For example, another genome-length (24S) mRNA could be transcribed that would have multiple initiation and termination sites for translation similar to the mRNA of RNA phages (13). Alternatively, subgenomic-size mRNA's could be transcribed, as they are in vesicular stomatitis virus-infected cells (9). Whatever the case, IPNV probably has a unique mechanism to synthesize three size classes of polypeptides, using mRNA transcripts from two high-molecular-weight dsRNA genome segments.

ACKNOWLEDGMENTS

We thank J. Wood for helpful comments on the manuscript.

This investigation was supported by the National Research Council of Canada.

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