Rubella Virus: Structural and Non-structural Proteins

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(Accepted 6 February 1984)

SUMMARY

Rubella virus was rapidly concentrated and purified using polyethylene glycol 6000 as the precipitating agent. Electrophoresis in slab gels defined three structural proteins present in equimolar amounts, with mol. wt. of 59000 (E1), 43000 to 48000 (heterogeneous E2) and 34000 (core protein, C). E1 and E2 were glycosylated; different distributions of labelled carbohydrates within the broad band of E2 indicated that the slower migrating region was enriched in complex oligosaccharides. In infected cells, the counterparts to E1 and E2 were labelled with [3H]mannose and both migrated in gels as sharp bands, indicating that the heterogeneity observed in virion E2 was produced during virus maturation. After radioimmunoprecipitation of infected cell extracts with convalescent rubella serum, the intracellular equivalents of E1, E2 and C were readily defined in gels, as well as several putative non-structural proteins. Four of these were defined more clearly and without resort to immunoprecipitation by labelling with [35S]methionine during hypertonic treatment of infected cells at 24 h; their mol. wt. were 200000, 150000, 87000 and 75000. Pulse–chase experiments under these conditions showed that the largest (ns200) was apparently cleaved to ns150.

INTRODUCTION

Rubella virus (RV), the only member of the Rubivirus genus in the family Togaviridae, is a spherical enveloped virus about 60 nm in diameter (Holmes et al., 1969) containing a single strand of RNA which is infectious and hence of positive polarity (Hovi & Vaheri, 1970). Until recently, several discrepancies were obvious in reports on the size and number of RV structural proteins. Although most of the early data indicated that three major structural proteins were present (Vaheri & Hovi, 1972; Payment et al., 1975a; Ho-Terry & Cohen, 1980; Trudel et al., 1982), some reports claimed additional minor proteins (Liebhaber & Gross, 1972; Bardeletti et al., 1975), and one report was at variance with all other data (Van Alstyne et al., 1981). The most recent reports on the structural proteins of RV (Ho-Terry & Cohen, 1982; Waxham & Wolinsky, 1983; Oker-Blom et al., 1983) have indicated that it is composed essentially of only three structural proteins, namely two envelope glycoproteins, E1 (mol. wt. 55000 to 62000) and a heterogeneous E2 (42000 to 54000), and a core protein C (33000 to 38000). There have been only four reports on the detection of intracellular RV proteins. Oker-Blom et al. (1983) identified the intracellular counterparts to the structural proteins but their results differed from those of Pope & Van Alstyne (1981). Several apparently RV-specific polypeptides were detected by Chantler (1979) and Chantler & Tingle (1980) but because the authors were unable to identify the structural proteins, no further characterization could be carried out.

We present in this paper electrophoretic analyses of the structural proteins of RV purified by rapid concentration using precipitation with polyethylene glycol (PEG) 6000 and a single ratezonal sedimentation. We show that the intracellular counterparts of the virion proteins were detectably labelled in infected cells with [3H]mannose and [35S]methionine, and we resolved also in slab gels several other virus-specified proteins of which one appeared to be a large precursor of non-structural protein(s).
METHODS

Cells and virus. Vero cells were grown in Medium 199 (M199) containing 6% heat-inactivated foetal calf serum (FCS) and after infection were maintained with Eagle’s minimum essential medium (MEM) containing 0.1% bovine serum albumin (BSA) and 14 mM-HEPES. The Putnam strain of RV was supplied by Commonwealth Serum Laboratories, Melbourne, and subsequently mycoplasma-free virus pools were prepared from culture fluids of infected Vero cells.

Plaque assay. Vero cell monolayers in 60 mm Petri dishes were infected with 0.2 ml of virus suspension in Hanks’ balanced salt solution (BSS) containing 0.1% BSA. The adsorption period was 1 h at 37°C; each culture was then washed with Hanks’ BSS and covered with 5 ml of overlay medium (M199, 5% FCS, 28 mM-HEPES, 0.05% DEAE-dextran and 1% Noble agar). After 6 days at 37°C in a 5% CO₂ incubator, cultures were stained with 0.0075% neutral red in Hanks’ BSS for 3 h.

Virus labelling, concentration and purification. Cells grown in 150 cm² tissue culture flasks (Costar) were infected at an m.o.i. of 1. Virions were labelled during growth at 37°C by incubation with radioactive amino acids or carbohydrate in 40 ml (per flask) of maintenance medium containing 1/10 normal concentration of amino acids or carbohydrate between 24 and 48 h post-infection. For labelling the polypeptide backbones, 5 μCi/ml [35S]methionine or 10 μCi/ml 3H-amino acid mixture was added, or 10 μCi/ml [3H]mannose, [3H]glucosamine or [3H]galactose was added for labelling glycoproteins. Virus was purified at 4°C from 80 to 160 ml of harvested culture fluids which were first centrifuged at 10000 g for 10 min; PEG 6000 was then added to a final concentration of approx. 7%. After 1 to 3 h, the virus was pelleted at 10000 g for 30 min and resuspended in 2 ml Tris-saline (0.012 M-Tris-HCl pH 6.8, 0.12 M-NaCl) containing 0.1% BSA. The virus suspension was layered onto a 15 to 30% (w/v) sucrose gradient prepared with Tris-saline and virions were purified by rate-zonal sedimentation. Peak virus fractions were identified by assays of cts/min, and by titration of haemagglutinin (HA) employing an SDS-containing discontinuous buffer system (Laemmli, 1970). After electrophoresis, gels were processed for fluorography (Bonner & Laskey, 1974).

Preparation of labelled cells for radioimmunoprecipitation. Vero cell monolayers in 16 mm wells of a multidish Disposo Tray (Linbro) were infected at 37°C with RV at an m.o.i. of 5 to 10. At 42 h, cells were treated with actinomycin D (3 μg/ml) in MEM/BSA containing 1/50 normal concentration of methionine or 1/10 normal concentration of glucose; at 45 h each culture was re-fed with 250 μl of glucose-depleted medium containing [3H]mannose (200 μCi/ml) or of methionine-deficient medium containing [35S]methionine (100 μCi/ml). After 2 to 3 h, labelled cells were washed with phosphate-buffered saline and dissolved in 0.4 ml lysis buffer.

Radioimmunoprecipitation of virus-specified proteins. Clarified cell extracts were prepared according to the method of Wechsler et al. (1979). Aliquots of 100 μl containing labelled cell extracts, or labelled virion proteins, and 2 mM-phenylmethylsulphonyl fluoridyl (PMSF) were immunoprecipitated using Protein A-Sepharose as described by Wright et al. (1981). A convalescent rubella serum was used as a source of virus antibodies. The immunoprecipitated proteins were eluted and collected from the final gel pellet by addition of 20 μl of 3% SDS followed by heating at 100°C for 2 min. The residual proteins were extracted from the pellet with a further 20 μl 3% SDS and the supernatants combined.

Polyacrylamide gel electrophoresis. Protein samples adjusted to 2% SDS were analysed in polyacrylamide gels employing an SDS-containing discontinuous buffer system (Laemmli, 1970). After electrophoresis, gels were processed for fluorography (Bonner & Laskey, 1974).

Isotopes and chemicals. Radioactive materials supplied by Amersham (Australia) were D-[2-3H]mannose (13.4 Ci/mmol), L-[35S]methionine (1280 Ci/mmol), D-[6-3H]glucosamine (10-25 Ci/mmol), D-[1-3H]galactose (10-7 Ci/mmol), [14C]Methylated protein mixture (code CFA.626) and a tritiated amino acid mixture (code TRK.440). Actinomycin D was a gift from Merck, Sharp & Dohme (Sydney). Protein A-Sepharose CL-4B used in radioimmunoprecipitation was obtained from Pharmacia and PEG 6000 was supplied by Koch-Light Laboratories.

RESULTS

Virus purification

The latent period in Vero cells was 8 h, as noted by Payment et al. (1975b). At an m.o.i. of 1 to 10, maximum virus yields were attained at approx. 24 h post-infection, prior to the onset of significant c.p.e. When the m.o.i. was reduced, yields were significantly delayed and cells exhibited prominent c.p.e. at the time of maximum virus production (approx. 72 h). Virions were precipitated from the serum-deficient medium by PEG 6000 with 30 to 60% recovery of infectivity. As RV has similar sedimentation properties to alphaviruses (Laude, 1979),
concentrated virus suspensions were purified as for alphaviruses (Strauss et al., 1968) by rate-zonal sedimentation in 15 to 30% (w/v) sucrose gradients. Under these conditions a single sharp and coincident peak was obtained for radioactively labelled virus, infectivity and HA (Fig. 1).

**Structural proteins**

In preparations of purified virions labelled in amino acids, three structural proteins were detected, as shown in Fig. 2. Their mol. wt., calculated by reference to $^{14}$C-labelled standard proteins, were 59K (E1), a complex ranging from 43 to 48K (E2), and 34K (C); the virus specificity was confirmed by radioimmunoprecipitation (Fig. 2a, lane 3). Radioactive carbohydrate labelled only E1 and E2 (Fig. 2a, lanes 2 and 4) thus confirming that they correspond to the envelope glycoproteins of similar mol. wt. described previously by some authors (Vaheri & Hovi, 1972; Payment et al., 1975a; Ho-Terry & Cohen, 1980; Trudel et al., 1982; Oker-Blom et al., 1983; Waxham & Wolinsky, 1983). Longer exposure times of the fluorograms did not reveal the VP24 and VP19 reported by Van Alstyne et al. (1981). Densitometer analyses from fluorograms of separated RV proteins labelled with tritiated mixed amino acids indicated that the three structural proteins were present in approximately equimolar amounts (results not shown). This finding is in variance with that of Vaheri & Hovi (1972), but their ratios of 5:1:5 for E1:E2:C were based on low radioactive counts eluted from cylindrical gel slices.
Identification of intracellular counterparts to RV structural proteins

The electrophoretic profiles of amino acid-labelled proteins in mock-infected cells and RV-infected cells were virtually indistinguishable (see Fig. 4a, lanes 2 and 3), and initial identification of structural proteins in cells depended on the use of [3H]mannose as label. Under these conditions the host background was considerably decreased (Fig. 3, lane 1) and in RV-
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Fig. 3. Fluorogram of an 8% polyacrylamide gel showing intracellular counterparts to E1 and E2 of RV labelled with $^{3}$H]mannose. Infected Vero cells were labelled at 2 days post-infection and cytoplasmic extracts were electrophoresed before and after immunoprecipitation. Lane 1, mock-infected cells; lane 2, RV-infected cells; lane 3, mock-infected cells after immunoprecipitation; lane 4, RV-infected cells after immunoprecipitation; lanes 5 and 6, proteins of purified RV labelled with $^{3}$H]mannose and $^{35}$S]methionine, respectively. GP59(E1) and GP43(E2), indicated by arrowheads, are the intracellular counterparts to E1 and E2, respectively.

infected cells two prominent bands were resolved at 59K and 43K (Fig. 3, lane 2). The 59K glycoprotein clearly corresponds to E1. However, no diffuse band corresponding to the virion E2 was observed; instead, a sharp dense band migrating faster than most of the diffuse virion E2 band appeared to be the intracellular counterpart. Both the 59K and 43K glycoproteins were immunoprecipitated, confirming their identity as RV-specified proteins (Fig. 3, lane 4). After immunoprecipitation of $^{35}$S]methionine-labelled infected cytoplasm, three prominent proteins were resolved (Fig. 4a, b, lanes 4 and 8, respectively), two of which correspond to the 59K and 43K species previously seen and a 34K protein which corresponds in size to core protein. Because the extent of glycosylation of the intracellular equivalents has not been defined relative to the virion glycoproteins, we propose that they be provisionally designated GP59(E1) and GP43(E2). There is no reason to doubt the nature of the 34K intracellular protein which was specifically immunoprecipitated (compare Fig. 4a, lanes 2 and 3 with Fig. 4b, lanes 7 and 8) and hence it is designated C. Two minor proteins labelled with $^{35}$S]methionine were
Fig. 4. Fluorograms of RV-specified polypeptides in infected Vero cells identified by radioimmunoprecipitation. Uninfected and RV-infected cells were labelled for 3 h with [35S]methionine or [3H]mannose at 2 days post-infection and cytoplasmic extracts were electrophoresed in discontinuous slab gels. (a) 8% gel. Lane 1, 14C-labelled mol. wt. markers; lanes 2 and 3, [35S]methionine-labelled mock-infected and infected cell extracts, respectively; lane 4, the cell extract in lane 3 after immunoprecipitation; lane 5, structural proteins of purified RV labelled with [35S]methionine; lane 6, a longer exposure of lane 4. (b) 12% gel. Lanes 1 and 9 correspond to lanes 1 and 5, respectively, of part (a). Lanes 2 and 3, mock-infected and infected cell extracts labelled with [3H]mannose; lanes 4 and 5, the same cell extracts as in lanes 2 and 3, respectively, after immunoprecipitation; lane 6, envelope proteins of purified RV labelled with [3H]mannose; lanes 7 and 8, [35S]methionine-labelled mock-infected and infected cell extracts after immunoprecipitation.
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immunoprecipitated and detected migrating between GP59(E1) and GP43(E2) (Fig. 4a, lane 5), but these appear to be of host origin (Fig. 4b, lane 7); they do not contribute to the broad nature of virion E2 as they were not detected in electropherograms of [3H]mannose-labelled immunoprecipitated cytoplasm (Fig. 3, lane 4; Fig. 4b, lane 5).

Identification of intracellular RV non-structural polypeptides

[3H]Mannose-labelled infected cytoplasm analysed on a 12% discontinuous slab gel revealed in addition to GP59(E1) and GP43(E2) another glycoprotein of apparent mol. wt. 13000, designated GP13 (Fig. 4b, lane 3). This small glycoprotein has a relatively high carbohydrate content and was consistently seen only in profiles of infected cells labelled with [3H]mannose but it was not immunoprecipitated (Fig. 4b, lane 5). The absence of GP13 in immunoprecipitations may be due to a lack of antibody directed against the relatively small polypeptide or to a relatively high carbohydrate content that may hinder reaction of polypeptide determinants with antibody.

Several other virus-specified non-structural polypeptides smaller than C were detected by immunoprecipitation from [35S]methionine-labelled infected cytoplasm (Fig. 4b, lane 8). These have apparent mol. wt. of 27000 (ns27; also detected in Fig. 4a, lane 4), 20000 (ns20) and 17000 (ns17). Several virus-specified polypeptides larger than the structural proteins were also immunoprecipitated from infected cytoplasm (Fig. 4a, lanes 4 and 6; Fig. 4b, lane 8). The most prominent have apparent mol. wt. of 150000 (ns150) and 87000 (ns87); additional putative virus-specified polypeptides were also resolved with apparent mol. wt. of 110000 (ns110), 92000 (ns92), 77500 (ns78) and 75000 (ns75) (see also Fig. 5, lane 3). After a very long exposure of the immunoprecipitated lanes from fluorograms in Fig. 4(a) and Fig. 5, a further protein of mol. wt. 200000 (ns200) could also be detected, which was more clearly resolved in other immunoprecipitations of infected cytoplasm (results not shown).

We attempted to identify, earlier in infection, the larger RV-specified non-structural polypeptides in the cytoplasm by use of a hypertonic selective initiation block, as used previously by Chantler (1979) in RV-infected RK-13 cells. The technique relies on the viral mRNA having a greater affinity for ribosomes than does host mRNA under carefully selected hypertonic conditions, resulting in selective labelling of viral polypeptides. From infected cells labelled with [35S]methionine in the presence of 150 mM excess NaCl at 24 h, ns200, ns150, ns87 and ns75 were readily identified after electrophoresis in 8% gels (Fig. 5, lane 2). The specificity of these polypeptides was obvious because of either the intensity of label or because of the complete absence of comigrating polypeptides in extracts from mock-infected cells. These prominent bands coincide with some of the non-structural polypeptides previously resolved by immunoprecipitation of [35S]methionine-labelled infected cells (Fig. 5, lane 3); hence the status of these polypeptides as virus-specified has been confirmed by two independent methods. Pulse-chase experiments under hypertonic conditions in infected cells revealed that ns200 was apparently cleaved to ns150 (Fig. 6). At present the relationships to these larger species of the other non-structural polypeptides detected with excess-salt treatment have not been elucidated.

DISCUSSION

The apparent purity of the radiolabelled RV proteins in our electropherograms probably owes much to the simple and rapid method of virus concentration using PEG 6000 followed by rate-zonal sedimentation, which has been so successful in biochemical studies with alphavirus and flavivirus species (Westaway & Reedman, 1969; Grimes & Burge, 1971). The method helps overcome major problems associated with characterization of RV proteins, namely the fragility of the virus particle, contamination by cell proteins, and relatively low yields in cell culture. PEG 6000 has been used previously to concentrate RV infectious particles (Kanarek & Tribe, 1967) or RV HA (Trudel & Payment, 1980) but the structural proteins were not analysed in these reports. Trudel & Payment (1980) discarded the procedure in favour of hollow fibre ultrafiltration because of the large residue of total protein; this probably represented mainly serum proteins from the culture fluid which were precipitated by the high concentration (10%)
Fig. 5. Fluorogram of an 8% slab gel showing effects of hypertonic salt on translation of putative non-structural proteins specified by RV. Infected cells in 60 mm Petri dishes were infected at an m.o.i. of 10 and were treated with 150 mM excess NaCl for 30 min at 21 h. Cells were then labelled with 10 μCi/ml [35S]methionine for 3 h in the presence of the excess salt and harvested in 0-4 ml 2% SDS. Lanes 1 and 2, mock-infected and infected cell extracts, respectively, treated with hypertonic salt and electrophoresed without immunoprecipitation; lane 3, reference sample of immunoprecipitated proteins from infected cells labelled at 48 h with [35S]methionine using no salt treatment.

Fig. 6. Fluorogram of samples from a pulse–chase experiment analysed in an 8% slab gel. Cells were treated with 150 mM excess NaCl for 30 min at 24 h; cells were then pulse-labelled for 10 min with [35S]methionine and chased in the presence of hypertonic salt. The chase medium contained unlabelled methionine at 10 times the normal concentration. Lanes 1 to 6 represent increasing chase periods of 0, 5, 10, 20, 30 and 60 min.

From purified virus preparations we consistently identified three structural proteins in equimolar proportions using slab gels which yielded mol. wt. of 59K (E1), 43K to 48K (E2) and 34K (C). These sizes are in accord with some of the earlier estimates obtained using cylindrical gels (Vaheri & Hovi, 1972; Liebhaber & Gross, 1972; Payment et al., 1975a; Ho-Terry & Cohen, 1980). They are essentially identical with very recent mol. wt. estimates of 58K(E1), 42K of PEG 6000. Substitution of BSA for serum in culture medium and the lower concentration of PEG 6000 (about 7%) circumvented the precipitation problem.

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to 47K (E2) and 33K (C) by Oker-Blom et al. (1983), but are 3000 to 6000 less than those reported by Waxham & Wolinsky (1983). Both the latter groups, and Ho-Terry & Cohen (1982), resolved E2 into two or three distinct glycoproteins with apparently the same polypeptide backbone. Although we identified in slab gels the intracellular counterpart of E2 as a single sharp band labelled in [3H]mannose or [35S]methionine, some possibly variable glycosylation in our cell-virus strain system during virus maturation produced the observed heterogeneity in E2. Nevertheless, our results are in close agreement with those of Oker-Blom et al. (1983) in that the higher mol. wt. components of E2 are relatively low in mannose and high in galactose (compare their E2a) and the lower mol. wt. components are high in mannose and low in galactose (compare their E2b). Such labelling patterns are indicative of complex and simple oligosaccharides (Kornfeld & Kornfeld, 1980), respectively, in the two groupings of the E2 complex. The relatively lower incorporation or concentration in E1 of glucosamine and galactose (Fig. 2), or of glucosamine and galactose (Oker-Blom et al., 1983), or of fucose and glucosamine (Ho-Terry & Cohen, 1980), compared to E2, may indicate a smaller proportion of complex oligosaccharides in E1 relative to those in the heterogeneous E2. These conclusions are consistent also with the results of Waxham & Wolinsky (1983), who reported that E1 contained more mannose than glucosamine, while E2 contained more glucosamine than mannose; these authors did not, however, directly compare the proportions of each sugar between the respective glycoproteins.

The intracellular counterparts to the envelope glycoproteins were detected directly for the first time by [3H]mannose labelling of infected cells (Fig. 3), and all three counterparts to the RV structural proteins could be detected by immunoprecipitation of [35S]methionine-labelled infected cytoplasm with convalescent human serum (Fig. 4a, lane 4). The mol. wt. estimates of GP59(E1), GP43(E2) and intracellular C, compare well with those of the three intracellular species detected by Oker-Blom et al. (1983) by immunoprecipitation using hyperimmune rabbit antiserum. As noted in the Introduction, there have been reports from other laboratories describing intracellular RV polypeptides but these were either related to RV structural proteins of anomalous mol. wt. (Pope & Van Alstyne, 1981) or could not be related directly to RV structural proteins (Chantler, 1979; Chantler & Tingle, 1980).

The maximum coding content of the RV genome (mol. wt. 3·2 × 10^6, estimated by Sedwick & Sokol, 1970) is probably slightly in excess of 300 000 daltons of protein. The largest detected product, ns200, represents most of the coding potential; it is expressed early and is post-translationally cleaved to yield ns150 (Fig. 6). Because of its large size, it seems unlikely that ns200 codes for the structural proteins; it may be the uncleaved precursor to non-structural proteins required for replication. It is of interest that the alphaviruses Semliki Forest virus and Sindbis virus both specify a large non-structural precursor protein (p200–230) which is cleaved to smaller products, initially to p150–155, and finally to three non-structural proteins, mol. wt. range 60 000 to 90 000 (Schlesinger & Kääriäinen, 1980). Of the other possible RV non-structural proteins, ns110, ns92, ns75 and ns27 may be equivalent to p109, p92, p75 and p28/29 reported by Chantler (1979) to be labelled under hypertonic conditions at 72 to 78 h in RV-infected RK-13 cells, but none of the latter was detected at 0 to 16 h or 24 to 40 h post-infection. Other prominently labelled products reported in salt-treated infected RK-13 cells were p46, p40 and p32.5; a p78 (compare our ns78) was also detected in infected human peripheral blood lymphocytes which support restricted replication of RV (Chantler & Tingle, 1980). Although some of the non-structural proteins detected by immunoprecipitation alone coincide with species reported by Chantler (1979) and Chantler & Tingle (1980), the possibility that they may be of host origin cannot be excluded.

Some of the non-structural RV proteins detected using hypertonic salt treatment and by immunoprecipitation may not be derived by cleavage from ns200 but may instead be related to the structural proteins. In some gel profiles of purified RV proteins, minor bands were reported which represent proteins of mol. wt. 105 000 to 109 000 and 95 000 to 96 000 (Ho-Terry & Cohen, 1980; Oker-Blom et al., 1983; Waxham & Wolinsky, 1983), and these appear to be disulphide-linked complexes of E1 dimers or hybrids of E1 plus E2, respectively (Waxham & Wolinsky, 1983). These complexes may be relevant to the appearance in some gels of infected cell extracts of ns110 and ns92 (Fig. 4a, lane 6) or of p109 and p92 (Chantler, 1979). However, ns110 and ns92
in our experiments were not always prominent and we have not observed any corresponding incorporation of \([^{3}H]\)mannose; hence, the occurrence of intracellular dimers, or hybrids of E1 and E2, seems unlikely. If the reported subgenomic 24S RNA in RV-infected cells (Oker-Blom et al., 1983) is equivalent to the alphavirus 26S mRNA which codes for the structural proteins, its maximum coding capacity would be in the vicinity of 130,000 daltons of protein. The unglycosylated forms of the RV envelope proteins (Oker-Blom et al., 1983) have a total mol. wt. of about 83,000 (compare our ns87) and with C comprise a total of about 116,000 (compare our ns110). Tryptic peptide analyses are obviously required to establish any possible relationships between ns110 or ns87 and the structural proteins. A further analogy with alphaviruses is the occurrence of GP13 (Fig. 4b, lane 3) which is comparable to the intracellular E3 of Sindbis virus and which is also not incorporated into the virion (Garoff et al., 1982). However, there are no alphavirus counterparts to the small non-structural polypeptides ns27, ns20 and ns17 (Fig. 4b, lane 8) which are immunoprecipitated by RV convalescent serum.

The results discussed above provide further evidence that RV shares many properties with the alphaviruses, fully justifying its inclusion within the Togaviridae. RV has a similar sedimentation coefficient and buoyant density (Laude, 1979), and the mol. wt. of E1, E2 and C resemble closely those of corresponding proteins of Sindbis virus, which also occur in equimolar amounts (Garoff et al., 1982). Both viruses specify a large non-structural protein of mol. wt. 200,000 which is post-translationally cleaved to yield a product of approx. 150,000 mol. wt. However, RV does not achieve selective switch-off of host protein synthesis and the large number of non-structural proteins indicates possibly more complex pathways of post-translational cleavage. Elucidation of these pathways should soon be possible.

This project was supported in part by the National Health and Medical Research Council of Australia.

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(Received 8 December 1983)