The Structural Proteins of Rabies Virus and Evidence for their Synthesis from Separate Monocistronic RNA Species

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SUMMARY

Purified preparations of the CVS strain of rabies virus, which were labelled during the infectious growth cycle with different isotopes or labelled in vitro by iodination or acetylation, contained five major proteins, L, G, N, M₁ and M₂, when examined by polyacrylamide gel electrophoresis (PAGE). The major surface glycoprotein, G, could be separated into two components, G₁ and G₂, in some PAGE systems; they were present in about equimolar amounts and had apparent mol. wt. of 70.5 × 10⁴ and 65 × 10⁴, respectively. The virus nucleocapsid (ρ = 1.28 g/ml) could be isolated after detergent treatment of purified virus. It contained the virus RNA, the major nucleocapsid protein, N (mol. wt. 58.5 × 10⁴), and small amounts of a large protein, L (mol. wt. 170 × 10⁴). Two membrane proteins, M₁ (mol. wt. 39.5 × 10⁴) and M₂ (mol. wt. 25 × 10⁴), were also observed. Chromatography of dissociated rabies virus in agarose columns with guanidine hydrochloride did not resolve any additional virus structural proteins. Two-dimensional peptide map analysis of iodinated structural proteins indicated that they were unique gene products and not derived from a precursor polypeptide by cleavage. The peptide maps of the two glycoproteins, G₁ and G₂, appeared identical. The approximate number of protein molecules per virion has been determined. Rabies virus-directed protein synthesis in BHK₂₁ cultures was detected as early as 6 h p.i. and all the proteins were present 12 h p.i. Additional non-structural virus-specific proteins were not observed. The NaCl hypertonic shock procedure, which differentially inhibits polypeptide chain initiation in different classes of mRNAs, was used to inhibit the synthesis of the G and M₁ proteins relative to the others selectively. All the rabies virus proteins were synthesized simultaneously following release from hypertonic treatment, suggesting that there are independent polypeptide chain initiation sites for the synthesis of each of the rabies proteins and that each protein is derived via translation of monocistronic mRNA species.

INTRODUCTION

Rabies virus is a member of the *Rhabdoviridae* family and it has been classified as a negative-strand virus primarily because the virion RNA was non-infectious (Sokol, 1975) and because transcriptase activity was associated with purified virions (Kawai, 1977; Flamand *et al.* 1978). Until recently, four major structural proteins have been identified in purified

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virus preparations— a nucleocapsid protein, N; two envelope proteins, M₁ and M₂; and a surface glycoprotein G, which in some strains can be separated into two species (Sokol et al. 1971; Neurath et al. 1972). Madore & England (1977) have been able to detect these proteins in virus-infected cells and have identified the presence of a large protein, L (mol. wt. 190 × 10³), in purified virions and in infected cell extracts. Kawai (1977) was also able to detect the L protein in purified virus and associated its presence with virion transcriptase activity. Two minor polypeptides, P₄₀ and P₄₂, which comprise about 2 to 4% of the total virion protein, have been observed in some virus preparations. Polypeptide P₄₂ was shown by peptide map analysis to be identical to BHK₂₁ cell actin. The origin of P₄₀ was not clear. It was suggested that it was not a cleavage product of the virion G or N proteins since its tryptic peptide map was different (Naito & Matsumoto, 1978).

Flamand & Delagneau (1978) have provided evidence that the gene order of rabies transcription on the virion RNA is 3'N-M₁-M₂-L5'. They were unable to establish firmly the location of G but suggested that if it was between M₂ and L then the transcriptional map of rabies virus would be very similar to that of vesicular stomatitis virus (VSV) whose gene order on the virion RNA is 3'N-NS-M-G-L5' (Abraham & Banerjee, 1976; Ball & White, 1976).

In this report the structural proteins of the CVS strain of rabies virus have been studied by several methods. Their synthesis in infected BHK₂₁ cell cultures was monitored and analysis of the results suggested that their synthesis resulted from the independent translation of monocistronic mRNA species.

**METHODS**

**Materials.** All radioisotopes used in this study were obtained from New England Nuclear Corp., Boston, Mass. Specially purified reagents for agarose and polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories, Richmond, Calif. Purified guanidine hydrochloride was from Heico, Inc., Delaware Water Gap, Pa. Other reagents used were crosslinked agarose (Sepharose CL-4B) from Pharmacia Fine Chemicals, Piscataway, N.J., and polygram (Macherey-Nagel) pre-coated TLC cellulose plates from Brinkman Instruments, Westbury, N.Y.

**Virus growth, isotopic labelling and purification of virus.** The Indiana serotype of VSV was grown in BHK₂₁ cells and purified by established methods (Obijeski et al. 1976). The challenge virus stock fixed strain of rabies virus, CVS (Kissling, 1958), was from a stock collection at the Center for Disease Control (CDC). It was plaque purified three times in confluent monolayers of BHK₂₁ cells. A working stock of virus was prepared by infecting a monolayer of BHK₂₁ cells (75 cm², about 10⁷ cells) with a single plaque isolate.

Confluent BHK₂₁ monolayers in roller bottles (490 cm²) or flasks (150 cm²) were infected at 0.05 to 0.1 p.f.u./cell and appropriate radioisotopes were added to label the virus proteins and/or RNA. Virus particles were concentrated and purified from infected culture fluids as described previously (Obijeski et al. 1976).

**Electrophoresis methods.** The separation of SDS-dissociated proteins by polyacrylamide gel electrophoresis (PAGE) using either an 8% (w/v) continuous phosphate buffer system (CONT-SDS), a 3 to 30% (w/v) linear polyacrylamide gradient gel system (GRADIENT-SDS), or a high-resolution PAGE system (DISC-SDS) has been described in detail (Esposito & Obijeski, 1976; Obijeski et al. 1976). Before electrophoresis, protein samples were boiled for 3 min in 5% (w/v) SDS, 3% (v/v) 2-mercaptoethanol (2-ME), 0.003% bromophenol blue and 10% (v/v) glycerol which contained either 0.01 M-sodium phosphate buffer, pH 7.0 (PDB), or 0.0625 M-tris-buffer, pH 6.8 (DDB). The location of labelled proteins after electrophoresis was determined as described by Obijeski et al. (1976).
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Isotopic labelling for infected cell analysis. Confluent BHK21 cell monolayers in 30 mm Petri dishes were washed twice with BSS and were either mock-infected or infected with about 20 to 50 p.f.u./cell; after 1 h absorption at 37 °C the monolayers were washed with BSS and 2 ml of MEM was added. At various times after incubation at 37 °C the medium was removed and 1 ml Earle’s BSS was added. For labelling under hypertonic conditions at 37 °C the NaCl concentration was raised to the desired level by adding 5 m-NaCl for 15 min, and the cells were pulse-labelled for 1 h with either 10 μCi/ml 14C- or 100 μCi/ml 3H-amino acid mixtures.

In some experiments, infected BHK21 monolayers (30 mm Petri dish) were treated with 210 mM excess NaCl and incubated for 40 min at 37 °C in this hypertonic medium to cause complete ribosome “run-off” and to prevent ribosome re-initiation (Saborio et al. 1974). After this hypertonic treatment the cultures were washed three times with isotonic BSS (110 mM-NaCl) and methionine-free MEM was added. Each culture was pulse-labelled with 100 μCi ml 35S-methionine for various times after being placed in isotonic medium and the label was chased for 30 min at 37 °C in MEM containing a 1000-fold excess of unlabelled methionine. After the pulse-chase periods the medium was removed and the cultures were lysed immediately with 0.3 ml of DDB. The samples were transferred to small conical tubes (1.5 ml) and sonicated (Lamb et al. 1976).

Chromatography of rabies virus proteins. A purified preparation of unlabelled rabies virus (approx. 10 mg total protein) was mixed with about 5 × 10^6 ct/min of 14C-amino acid-labelled virus and the mixture was washed once with 10 vol. of cold acetone at -20 °C for 1 h to remove lipid. The virus preparation was collected by centrifugation and was dissociated in 1.5 ml of 0.05 M-tris-buffer, pH 7.6, 0.01 M-EDTA, 0.1 M-dithiothreitol (DTT), 0.5 %, (v/v) NP40 and 8 M-guanidine hydrochloride by incubating at 60 °C for 1 h (Green & Bolognesi, 1974; Fleissner, 1971). Bromophenol blue (0.1 %) was added as a void volume marker and the mixture was applied (via a 2 ml injection loop) to a 4 % agarose column (Sepharose CL-4B; 120 × 1.5 cm) previously equilibrated with 6 M-guanidine hydrochloride, 0.01 M-DTT and 0.02 M-sodium phosphate buffer (pH 6.5). The flow rate was adjusted to 2 ml/h by gravity flow and 1 ml fractions were collected. The elution of the virus proteins was determined either by monitoring the radioactivity in 0.05 ml samples of each fraction or by monitoring absorbance at 280 nm with a Beckman Model 25 recording spectrophotometer. Guanidine hydrochloride was removed from pooled samples by dialysis and the samples were concentrated by lyophilization. A portion of each sample was used for PAGE analysis.

Iodination and acetylation of virus proteins. Purified virus preparations were acetylated with 3H-acetic anhydride by the method of Montelaro & Rueckert (1975). Iodination of NP40-treated virus was done as previously described by Obijeski et al. (1976).

Tryptic peptide mapping. Iodinated rabies virus was dissociated with DDB and the proteins were separated by DISC-SDS PAGE. After electrophoresis the proteins were located by staining and cut from the gel. Each gel section was pulverized by extrusion through 14- and 18-gauge needles into 2 ml of 0.02 M-phosphate buffer (pH 7.0) containing 2 % SDS and 0.5 % 2-ME. The gel slurries were stirred overnight to elute the proteins and, after removing the acrylamide pulp by centrifugation, the proteins and 20 μg myoglobin carrier were concentrated with 2 vol. of methanol acidified with 1 % (v/v) glacial acetic acid. After standing for 6 h at -20 °C, the proteins were collected by centrifugation at 25000 rev/min for 30 min at 4 °C, washed twice with methanol and resuspended in 1 % (w/v) ammonium bicarbonate, pH 8.1, and 0.001 M-calcium chloride. The proteins were digested with 10 μg/ml trypsin (TPCK-trypsin, Worthington Biochemical Corp., Freehold, N.J.) for 16 h at 37 °C. The samples were concentrated by lyophilization and two-dimensional tryptic fingerprints were prepared on thin-layer cellulose plates as described by Inglis et al. (1976).
RESULTS

Structural proteins of the CVS strain of rabies virus

Early reports indicated that rabies virus contained four major structural proteins: G, N, M₁ and M₂ (Sokol et al. 1971). Another report indicated that the glycoprotein G could be resolved into two components, G₁ and G₂ (Neurath et al. 1972). Recent studies have shown the presence of another protein, L, as well as two additional minor proteins, P₄₂ and P₄₀.
Fig. 2. Electropherograms of in vitro labelled rabies virus structural proteins. A purified preparation of rabies virus was divided into three equal portions. One was acetylated directly at 20 °C for 30 min by the addition of 5 mCi of \(^{3}H\)-acetic anhydride. Another portion was treated with 1% (v/v) SDS before adding acetic anhydride. The final portion was iodinated by treating it with 2% (v/v) NP40 and 1 mCi \(^{125}I\)-iodine in the presence of lactoperoxidase and hydrogen peroxidase. Virus proteins acetylated (---) without prior SDS-treatment (a) or with SDS-treatment (b) were mixed separately with \(^{14}C\)-amino acid-labelled virus proteins (O-O) and were solubilized with PDB. The virus proteins were resolved by electrophoresis in 8% CONT-SDS gels for 16 h at a constant current of 4 mA/gel. Virus proteins labelled with iodine (c) were also resolved by CONT-SDS gel electrophoresis.

(Kawai, 1977; Madore & England, 1977; Naito & Matsumoto, 1978). Therefore, it was important to establish the virus structural proteins of the CVS strain of rabies virus used in this study.

Differentially labelled preparations of rabies virus and VSV-Indiana strain were dissociated and co-electrophoresed to compare the proteins of the two viruses and to estimate the mol. wt. of the rabies proteins. Three gel systems, CONT-SDS, DISC-SDS and GRADIENT-SDS (Esposito & Obijeski, 1976) as described in the Methods, were used. These particular gel systems were used because some proteins of similar mol. wt. might be better separated by one gel system but not by another. Four major proteins were detected in each gel system as well as small amounts of a very large protein (Fig. 1); the proteins are given the lettered designations L, G, N, M₁ and M₂, in order of decreasing mol. wt. to fit the established nomenclature for rhabdovirus proteins (Wagner et al., 1972). A split or separation of the G protein was also observed in the DISC-SDS gel system. This separation of the G protein into two species (identified as \(G₁\) and \(G₂\)) was usually observed when rabies proteins were
resolved by this gel system. The separation of these two proteins was more clearly defined in either stained gels or in gels prepared for autoradiography (unpublished data). Another report has also provided the same evidence for two components of the G protein (Neurath et al. 1972). Although not shown here, both the G1 and G2 proteins could be labelled with glucosamine or fucose and are therefore identified as glycoproteins.

To identify further any virus protein not labelled or resolved by in vivo labelling techniques, we examined two in vitro isotope labelling procedures. The first method, a radioacetylation procedure, was applied to intact as well as SDS-disrupted virions (Fig. 2 a and b). The second method was lactoperoxidase-catalysed iodination of NP40-treated virus particles (Fig. 2 c). Rabies virus acetylated after disruption with SDS contained several minor unidentified radioactive peaks; although the major virus proteins were easily identified. The acetylation procedure caused a minor decrease in protein mobility, which may be accounted for by the addition of acetyl groups to reactive amino groups during the acetylation procedure (Montelaro & Rueckert, 1975). The iodination procedure labelled all the virus proteins to high specific activity (Fig. 2 c); these proteins could be used for subsequent peptide mapping studies (see below).

To determine if any other proteins were present in purified rabies virus which were not resolved by SDS–PAGE and to develop a semi-preparative method for isolating rabies proteins, purified rabies virus was dissociated with guanidine hydrochloride (GuHCl) and the proteins chromatographed through a 4% agarose column (Fig. 3). A single major peak (no. 2) and three minor peaks (no. 1, 3, 4) were reproducibly found in the chromatogram. Peaks 1, 2, 3, and 4 from the elution profile were pooled separately, dialysed free of GuHCl.

Fig. 3. Gel filtration of rabies virus proteins in agarose columns containing guanidine hydrochloride (GuHCl) and dithiothreitol (DTT). A purified preparation of rabies virus (10 μg), suspended in 1.5 ml of 0.05 M-tris buffer (pH 7.6), 0.01 M-EDTA, was mixed with 14C-amino acid-labelled virus (approx. 5 x 10⁵ ct/min) and was dissociated with 0.5% (v/v) NP40, 0.1 M-DTT and 8 M-GuHCl at 60 °C for 1 h. The dissociated mixture was applied with an injection loop to a Sepharose CL-4B column (1.5 x 120 cm), which was previously equilibrated in 0.02 M-sodium phosphate buffer (pH 6.5), 0.01 M-DTT and 6 M-GuHCl and the proteins were chromatographed at a flow rate of 1 ml/30 min. Fractions (1 ml) were collected and the virus proteins were detected by monitoring the 14C-radioactivity in 0.1 ml aliquots of each fraction. The numbers on the elution profile (i.e. 1, 2, 3, 4) indicate the areas which were pooled for subsequent gel electrophoresis analysis.
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Fig. 4. Gel electrophoresis of rabies virus proteins isolated by guanidine hydrochloride gel chromatography. Pooled fractions 1, 2, 2 and 4 from the elution profile in Fig. 3 were dialysed free of GuHCl; the proteins were recovered by precipitation with cold acetone. The proteins were dissociated with PDB and were resolved by 8% CONT-SDS gel electrophoresis. Letters (L, G, N, M₁, M₂) indicate the positions of the structural proteins of purified rabies virus electrophoresed in parallel run gels.

and analysed for their protein content by PAGE (Fig. 4). The fastest eluting peak from the column (no. 1) contained only the L protein; peak 2 contained both the G and N proteins as well as some minor proteins. Protein M₁ was the major protein species in peak 3 and only protein M₂ was found in peak 4. These results indicate that chromatography through agarose may provide an effective preparative method for isolating the major protein species of rabies virus. However, the separation of the G and N proteins in peak 2 will require additional purification; this might be accomplished by selection of a different gel matrix.

Mol. wt. estimates and the relative contribution of each protein species to the total protein content of the virus are detailed in Table 1 (see also Bishop & Roy, 1971; Obijeski et al. 1976). The determination of the approximate number of virus proteins is based upon the following three assumptions: (1) that the estimate of the mol. wt. of the virus proteins are correct; (2) that the virus particle contains one copy of the genome RNA; and (3) that the total mol. wt. of rabies RNA is $3.8 \times 10^6$ (Holloway & Obijeski, 1980).
To establish the unique nature of rabies virus structural proteins, iodinated rabies virus proteins were separated by PAGE and digested with trypsin. Two-dimensional fingerprints prepared for the peptides of each protein were clearly different by visual inspection (Fig. 5). The fingerprints of the two glycoproteins, G1 and G2, appeared to be identical, indicating that the protein moiety of both these glycoproteins are the result of the same gene product. These peptide maps were reproducible in three separate trials and provide evidence that there are at least five separate gene products of rabies virus messenger RNA.

Rabies virus nucleocapsid proteins

Previous reports have shown that the nucleocapsid (NC) of rabies virus, released by detergent treatment and purified by CsCl density gradient centrifugation, contains the virus RNA and the N protein (Sokol et al. 1969). Since a large protein, L, was always found in our purified virus preparations (see Fig. 1 and 2), we were interested in establishing whether or not this protein was associated with the NC; a large protein species was found in nucleocapsid preparations of another rhabdovirus, VSV (Wagner, 1975). A purified preparation of rabies virus, doubly labelled with 35S-methionine and 3H-uridine, was divided into three equal aliquots and each treated with either NP40, DOC or a mixture of the two detergents. Each aliquot was loaded on to KT:GLY gradients (Obijeski et al. 1976) and after 24 h centrifugation at 41,000 rev/min it was found that essentially all the 3H-label was present in a single band (ρ = 1.28 g/ml) together with about one-third of the 35S-label (Fig. 6). The rest of the 35S-label was recovered as a broad band at a density of about 1.10 to 1.20 g/ml. To identify the proteins associated with material from both regions of each gradient, the indicated fractions were pooled separately and dialysed to remove the potassium tartrate. After concentration the proteins in each fraction were resolved by PAGE (Fig. 7). The proteins associated with the virus RNA (fraction I) after treatment with DOC alone or DOC and NP40 were the L and N proteins. Fraction II material contained the virus glycoprotein G and the two membrane proteins M1 and M2. Fraction I, after NP40 treatment, contained all the virus structural proteins except G; fraction II contained not only the glycoprotein but also about 60% of protein M1. The two proteins most closely associated with the virus RNA were, therefore, the L and N proteins. Although not shown here, the NC isolated after DOC treatment was resistant to pancreatic ribonuclease digestion (50 μg/ml for 30 min at 37°C); the isolated NC of VSV is also resistant to ribonuclease digestion (Hefti & Bishop, 1975).

Intracellular protein synthesis in rabies virus-infected BHK21 cells

Since the CVS strain of rabies virus did not appreciably reduce the accumulation of radioactive precursors into host-cell proteins (our unpublished data), it was necessary to eliminate this inherent difficulty by other methods before examining virus-specific protein synthesis. Neither the use of actinomycin (0.02 to 4 μg/ml) nor growth of cells at 32°C or 39°C was successful. However, the exposure of rabies virus infected BHK21 cells to hypertonic NaCl treatment before and during the labelling period, a procedure that selectively reduces the initiation of cellular protein synthesis, resulted in some degree of selective inhibition of cellular protein synthesis (Saborio et al. 1974; Madore & England, 1975, 1977; Nuss et al. 1977).
Fig. 6. Isolation of rabies virus nucleocapsids. A purified preparation of $^3$H-uridine (○—○) and $^{35}$S-methionine (△—△) labelled rabies virus in TSE buffer was treated for 20 min at 20°C with either 2% (v/v) NP40 (b) or 2% (v/v) DOC (c) or 1% (v/v) of NP40 and DOC (d) or was left untreated as a control (a). Each mixture was centrifuged for 24 h at 4°C and 41 000 rev/min (SW 41 rotor) in a combination KT:GLY gradient in TSE buffer. The distribution of radioisotope in each of thirty 0·4 ml fractions was determined by liquid scintillation counting of 0·1 ml aliquots. Densities (●—●) were determined by weighing 0·1 ml samples at room temperature.

1975; Lamb et al. 1976; Nuss & Koch, 1976). When rabies virus-infected cultures at 14 h p.i. were treated with 100 mM excess NaCl 15 min before and during a 1 h pulse, all five rabies virus proteins were detected (Fig. 8). The major peaks observed in the gel patterns of infected cells at this time corresponded to the major virus structural proteins; however, the
Rabies virus protein synthesis

Fig. 7. PAGE analysis of the proteins present in rabies virus fractions after detergent treatment and density gradient centrifugation. Fractions I and II from Fig. 6 obtained after either DOC, NP40, or DOC and NP40 treatment were separately pooled and dialysed free of potassium tartrate. Each fraction was concentrated with 10% (w/w) trichloroacetic acid, washed twice with acetone and dissociated with PDB. The proteins were resolved by CONT-SDS gel electrophoresis. A control virus preparation was electrophoresed in a parallel gel to mark the migration of the L, G, N, M1 and M2 proteins.

relative proportion of the glycoprotein was less in infected cells than in the virus. No additional virus-directed non-structural proteins were detected.

Having established that peaks corresponding to the virion proteins could be detected in rabies virus-infected cells (see Fig. 8), we sought a method which identified the kinetics of virus-specific protein synthesis relative to the inhibition of protein synthesis in the host cell. The double-isotope label difference analysis of infected and uninfected cells by PAGE has been found useful by others (Zweerink & Joklik, 1970; Hightower & Bratt, 1974; Lamb et al. 1976). We have used this method, coupled with hypertonic salt treatment, to examine rabies intracellular protein synthesis. At various times after infection mock-infected and infected cultures were treated for 15 min with 100 mM excess NaCl and were then pulsed for 1 h. To construct the difference analysis plot, 3H-amino acid-labelled infected cell extracts
Fig. 8. Electrophoresis of rabies virus-infected cell proteins. Two cultures of BHK21 cells (30 mm Petri dishes) were infected with rabies virus, and at 14 h p.i. the growth medium was removed. One culture was treated with 100 mM excess NaCl as described in Methods (a); the other was untreated (b). Both cultures were pulse-labelled for 1 h at 37 °C with 3H-amino acids and were prepared for gel electrophoresis. After CONT-SDS electrophoresis the gels were frozen, sliced into 1 mm pieces and the amount of isotope in each piece determined by liquid scintillation spectrometry. (c) An uninfected BHK21 culture pulse-labelled for 1 h with a mixture of 14C-amino acids. The positions of the L, G, N, M₁ and M₂ virus proteins electrophoresed in a separate gel are noted.

were co-electrophoresed with 14C-amino acid-labelled uninfected extracts. As shown in Fig. 9, a region of normalization (gel fraction 10 to 25), which contained no apparent virus proteins, was chosen. The total 3H-radioactivity (infected) was divided by the total 14C-radioactivity (uninfected) in this region of the gel and the 14C-radioactivity in each gel fraction was multiplied by this ratio to give the normalized 14C-radioactivity, which is operationally defined as host-cell background. The difference between the 3H-radioactivity in the infected cell and the normalized 14C-radioactivity is taken as virus-specific radioactivity (see Hightower & Bratt, 1974). Difference analysis plots of intracellular rabies protein synthesis at various times after infection are shown in Fig. 9.
Rabies virus protein synthesis

Fig. 9. Double-label difference analysis of rabies virus-infected BHK21 cell proteins. At 2 h, 6 h, 12 h, 15 h, 20 h, 24 h, 30 h, 40 h and 50 h p.i. the growth medium of infected and uninfected monolayer cultures (30 mm Petri dish) of BHK21 cells was removed and replaced with 2 ml BSS containing 5% dialysed foetal calf serum. After 15 min incubation at 37 °C (to decrease the intracellular amino acid pools) the concentration of NaCl in the medium was increased an additional 100 mM and after a further 15 min incubation (37 °C) in hypertonic medium 100 μCi 3H-amino acid mixture was added to the infected culture and 10 μCi of 14C-amino acid mixture was added to the uninfected cells. The radiolabelled medium was removed after a 1 h pulse at 37°C and the monolayers were washed twice with TSE buffer. The monolayers were solubilized with DDB, sonicated to shear the DNA and boiled for 5 min. The 3H-labelled infected cell proteins were co-electrophoresed with the 14C-labelled uninfected proteins in the DISC-SDS gel system. After electrophoresis each gel was sectioned into 1 mm segments and the distribution of each isotope in the gel was determined by liquid scintillation spectrometry. The 14C-radioactivity was normalized as described in the text, subtracted from the 3H-radioactivity in each gel slice and the difference between the two plotted (Δ).
Table I. Estimated number and mol. wt. of rabies virus proteins

<table>
<thead>
<tr>
<th>Protein species</th>
<th>Mol. wt. x 10^-3</th>
<th>Percentage of total virus protein</th>
<th>Mol. wt. of protein per virion (x 10^-6)</th>
<th>Number of molecules per virion</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>170.0</td>
<td>4.96</td>
<td>13.5</td>
<td>79</td>
</tr>
<tr>
<td>G_1</td>
<td>70.5</td>
<td>21.07</td>
<td>57.6</td>
<td>817</td>
</tr>
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<tr>
<td>N</td>
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<td>35.84</td>
<td>98.0</td>
<td>1675</td>
</tr>
<tr>
<td>M_1</td>
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<td>5.84</td>
<td>15.9</td>
<td>402</td>
</tr>
<tr>
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<td>25.0</td>
<td>10.56</td>
<td>28.9</td>
<td>1156</td>
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</table>

* Mol. wt. estimates were determined by co-migration on DISC-SDS gels using β-galactosidase, phosphorylase A, bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin, cytochrome c, and purified vesicular stomatitis virus (Indiana serotype) as standards.
† Percentage of each protein was calculated from four separate determinations of the area under each peak of stained gels scanned at 620 mm.
‡ Total virion protein was derived from a 72:1 ratio of virus protein to RNA and the estimate that the mol. wt. of the rabies genome is 3.8 x 10^6 (Holloway & Obijeski, 1980).
§ Number of protein molecules per virus particle was calculated by dividing the daltons of protein per virion by their mol. wt.

By this method the major nucleocapsid protein, N, was clearly resolved by 6 h p.i. At 12 h all the major structural proteins were apparent. The synthesis of the M_1 protein in infected cells was always greater than that found in purified virus (see Table I). At all time points studied, the amount of intracellular glycoprotein was always less than that found in the intact virus particle.

Selective inhibition of rabies virus protein synthesis by NaCl hypertonicity

To investigate the effect of NaCl hypertonicity on the synthesis of the various rabies virus structural proteins, infected BHK21 cell cultures were exposed to increasingly hypertonic NaCl in methionine-deficient medium at 18 h p.i. Fifteen minutes later, ^35S-methionine was added and after 1 h the labelling medium was removed and the cells were prepared for PAGE analysis. The data obtained after electrophoresis were normalized so that the peaks of the N protein were the same height (i.e. 100%). The synthesis of each virus protein was expressed, therefore, relative to the N protein (Fig. 10). It was evident that increasing the amount of NaCl led to a marked reduction in the amount of G and M_1 proteins relative to the N protein (Fig. 10). There was no detectable reduction of the M_2 protein. These results agree with those of Madore & England (1977), who also observed a reduction in the rate of synthesis of the G and M_1 proteins with increasing osmolarity.

Kinetics of intracellular and rabies virion protein synthesis following restoration of isotonicity

It is now well documented that the proteins of many animal RNA viruses are synthesized as large precursor polypeptides which are subsequently cleaved to give rise to the mature virus proteins (Korant, 1975). In contrast, with VSV, good evidence has established that the
Rabies virus protein synthesis

![Graph showing protein synthesis at different concentrations of NaCl](image-url)
Fig. 11. Gel electropherograms of rabies virus proteins synthesized in BHK21 cells after release from hypertonic NaCl treatment. Rabies virus infected BHK21 monolayer cultures (30 mm Petri dish) at 12 h p.i. were treated for 45 min at 37 °C with 210 mM excess NaCl. After hypertonic treatment the medium was removed, the culture washed three times and 1 ml methionine-free MEM added to each culture to restore isotonicity. Duplicate cultures were pulse-labelled with 100 μCi/ml 35S-methionine from 0 to 3 min, 0 to 20 min, 0 to 60 min, 3 to 6 min, 6 to 9 min, 9 to 12 min, 12 to 15 min and 15 to 20 min after restoring isotonicity. After the pulse each culture was chased with MEM containing a 1000-fold excess of unlabelled methionine. The chase medium was removed, the cells washed twice with TE buffer and the cell monolayer solubilized with PDB. Each preparation was sonicated to shear the cellular DNA and was heated to 100 °C for 5 min. The proteins were resolved by CONT-SDS gel electrophoresis. Letters indicate the position of 35S-methionine-labelled rabies virus structural proteins electrophoresed in a separate gel.
Rabies virus protein synthesis

Table 2. Percentage of total virus radioactivity in the structural proteins of rabies and VSV after release from hypertonic salt treatment

<table>
<thead>
<tr>
<th>Pulse period (min)</th>
<th>Rabies proteins (% of total)</th>
<th>VSV proteins (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L</td>
<td>G₂</td>
</tr>
<tr>
<td>0–3</td>
<td>4.5</td>
<td>15.6</td>
</tr>
<tr>
<td>3–6</td>
<td>4.7</td>
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<td>6–9</td>
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<td>0–60</td>
<td>1.9</td>
<td>18.9</td>
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<tr>
<td>Control§</td>
<td>1.7</td>
<td>23.4</td>
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</table>

* BHK₂₁ monolayer cultures (75 cm²) were infected with 20 to 50 p.f.u./cell of either rabies virus or VSV. At either 3 h (VSV) or 12 h (rabies virus) p.i. the cultures were treated with 210 mM excess NaCl for 40 min at 37 °C. The hypertonic medium was removed and isotonic conditions were established by quickly washing the cultures three times with BSS. The cultures were labelled with 100 µCi/ml ³⁵S-methionine in methionine-free MEM for various pulse periods after being placed in isotonic media. The labelling medium was removed and each culture was chased for 18 h at 33 °C in MEM containing a 1000-fold excess of unlabelled methionine. The culture fluids were collected and the extracellular virus was purified as described in Methods. Virus was dissociated with PDB and the proteins separated in 8 % CONT-SDS gels. After electrophoresis each gel was sliced into 1 mm pieces and the radioactivity in each was determined by liquid scintillation spectrometry.

† The percentage of radioactivity in each virus protein was computed from the total radioactivity in the virus proteins and represents the mean of two determinations.

‡ The rabies glycoproteins, G₁ and G₂, are not well resolved in CONT-SDS gels; therefore the percentages are of the total virus radioactivity contributed by G₁ and G₂ and are designated as G.

§ A control culture pulsed for 3 min without prior hypertonic salt treatment was included.

virus proteins are derived as primary gene products from separate monocistronic mRNAs without cleavage (Rose & Knipe, 1975; Freeman et al. 1977; Rhodes et al. 1977). There is no evidence that rabies virus proteins are synthesized similarly.

Preliminary experiments with amino acid analogues (canavanine, azetidine-2-carboxylic acid, p-fluorophenylalanine) and protease inhibitors (TPCK or TLCK) have failed to detect any large precursor proteins in rabies virus infected cells (unpublished data). Some suggestive evidence that separate mRNA species might be involved in rabies virus protein synthesis was obtained by examining the relative rates of protein synthesis in infected cells treated with increasingly hypertonic NaCl (see Fig. 10). The observation that the synthesis of the G and M₁ proteins were selectively suppressed relative to the other proteins indicated that these mRNA species had been differentially inhibited by the salt concentrations used and, in addition, their synthesis at the translational level was not under strict coordinate control. The restoration of isotonicity following hypertonic shock has been shown by others (Saborio et al. 1974; Nuss et al. 1975; Schochetman et al. 1977) and by us (unpublished data) to result in the immediate and synchronous re-initiation of protein synthesis. Consequently, if a large precursor(s) protein species existed in rabies virus-infected cells, the polypeptide nearest the ribosome initiation site (5'-terminus of the mRNA) would be the first synthesized and in a short pulse would be preferentially labelled relative to the protein nearer the 3'-end. To provide further independent evidence that rabies virus proteins are derived by separate initiation of monocistronic mRNA species (as in VSV) we utilized NaCl hypertonic salt treatment to follow the synchronous re-initiation of polypeptide chain synthesis. This was done by treating rabies virus-infected BHK₂₁ cells at 12 h p.i. with 210 mM-NaCl for 45 min, a treatment sufficient to inhibit cell and virus protein synthesis. Isotonicity was restored and the cultures were pulsed for various intervals with ³⁵S-methionine after reversal. The cultures were chased for 30 min and the protein species present were
resolved by PAGE. As seen in Fig. 11, rabies virus protein synthesis appeared to be simultaneously initiated regardless of the interval of the pulse period after restoring isotonicity. In a similar experiment the proteins labelled during the pulse intervals were chased for an additional 18 h, and the virus was purified from the extracellular fluids. The virus proteins were analysed by PAGE and the percentage of radioactivity in each protein relative to the total virus radioactivity throughout the gel was determined. Table 2 summarizes these results together with a control of VSV treated and processed by the same procedure. Most striking is that the percentage of each protein species remained essentially constant throughout the various pulse periods. Taken together these results provide strong evidence for the simultaneous re-initiation of each of the rabies virus proteins after restoration of isotonicity and indicate that rabies virus proteins are probably derived from separate monocistronic mRNA species without cleavage. The same positive results for VSV proteins, which are known to be derived by this mode of synthesis, further substantiated this notion (Table 2).

DISCUSSION

Several electrophoretic systems, labelling procedures as well as a chromatographic method, were used in this study to resolve the structural proteins of rabies virus (Fig. 1, 2, 3 and 4). Rabies virus proteins electrophoresed in the DISC-SDS gel system indicated that the virion glycoprotein could be separated into two species, G1 and G2, with apparent mol. wt. of 70.5 x 10^3 and 65 x 10^3, respectively (Fig. 1b). Two-dimensional peptide maps of iodinated rabies virus proteins established the L, N, M1 and M2 as unique gene products. The maps of G1 and G2 appeared to be identical and would suggest that they are derived from a common gene product. Differences between the two glycoproteins may reflect incomplete glycosylation or some degradation of the carbohydrate moiety during virus purification. Recently, Dietzschold et al. (1979) have also been able to separate the glycoproteins of the CVS-rabies virus strain into two components and have shown by ion exchange chromatography that their tryptic peptides were identical. Their study also provided evidence that the G2 protein contained less carbohydrate than G1, which might account for its faster electrophoretic mobility, hence its lower apparent mol. wt.

The G1 and G2 proteins were present in almost equimolar amounts in virus particles, whereas the number of molecules of the envelope protein M2 exceeded that of the M1 almost threefold. Only small amounts (about 80 copies/virion) of the L protein were found. The nucleocapsid protein, N, comprised about one-third of the total virus protein species (Table 1). Assuming a RNA-to-protein coding ratio of 10:1, it can be calculated that the five major rabies virus proteins (considering G2 as the glycoprotein) with a combined mol. wt. of 358,000 would account for more than 90% of the potential coding capacity of the rabies genome of mol. wt. 3.8 x 10^6 (Holloway & Obijeski, 1980). The remainder of the genome might be uncoded regions used for initiation and termination of protein synthesis.

By using appropriate hypertonic salt treatment to suppress cellular protein synthesis selectively, we were able to monitor the synthesis of rabies virus proteins in infected cultures. We observed no separation of the intracellular glycoprotein into two species as was seen in purified virus preparations. It was also noted that the relative proportion of cell glycoprotein was always less than that found in purified virus. This observation may reflect the sensitivity of the G protein to increasingly hypertonic NaCl (Fig. 10) and provides evidence for the lack of strict coordinate synthesis of this protein in rabies infected cells. Madore & England (1975, 1977) first utilized salt treatment of infected rabies virus cultures and were able to detect five proteins. They also observed in salt-treated cultures a virus-specific glycoprotein, gp78 (mol. wt. 78 x 10^3) which migrated slightly faster than the virion glyco-
Rabies virus protein synthesis

protein and could be chased into the mature virion glycoprotein (mol. wt. $80 \times 10^3$) when isotonicity was restored. They suggested that the hypertonic conditions might reversibly interfere with the processing of the virion glycoprotein.

The synthesis of rabies virus glycoprotein G and M$_i$ proteins was inhibited more than the synthesis of the other structural proteins when rabies virus infected cultures were exposed to increasingly hypertonic conditions. The synthesis of M$_a$ appeared to be most resistant (Fig. 10). Others have noticed the same effect with different rabies virus strains (Madore & England, 1977). The different sensitivities of the rabies virus proteins to hypertonic conditions suggest separate ribosomal binding sites for initiating protein synthesis. Similar studies with VSV indicated that the synthesis of the G and M proteins were most inhibited by increased hypertonicity (Nuss et al. 1975; Nuss & Koch, 1976). In this system the G protein is synthesized on membrane-associated polysomes whereas the M protein is translated on free, cytoplasmic polyribosomes (Knipe et al. 1977). There does not, therefore, appear to be a correlation between the site of synthesis of the individual proteins and their relative sensitivities to hypertonic salt treatment. The differential sensitivities to salt may, however, reflect the distance (that is, the number of nucleotides) between the 5'-capped end of the mRNAs and the initiation codons as suggested by Rose (1978).

Pulse-chase experiments after release from hypertonic salt suggest that rabies virus proteins are independently synthesized and do not arise from proteolytic cleavage of a precursor polypeptide(s) (Fig. 11, Table 2). It seems, therefore, that with the exception of kinetics, rabies virus protein synthesis in BHK21 cells is similar to that of VSV, although slower. Rabies virus proteins appear to be derived from the translation of independently transcribed monocistronic mRNAs. Indeed, analysis of rabies virus-infected cell RNA species indicates that the mRNA fraction of virus polysomes is heterogeneous (Holloway & Obijeski, 1980).

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REFERENCES


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