The Proteins of Sowthistle Yellow Vein Virus: Characterization and Location

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SUMMARY

SDS-polyacrylamide gel electrophoresis showed that purified sowthistle yellow vein virus (SYVV) contains four major and one minor structural polypeptides. The mol. wt. were estimated to be approx. 150000, 83000 (G), 60000 (N), 44000 (M1) and 36000 (M2). Covalently bound carbohydrate was detected in the 150000 mol. wt. species and the G protein.

Saponin and Nonidet P40 treatment removed the projections and membrane; however, the resultant bullet-shaped nucleocapsid structures, which contained N protein, were unstable and readily uncoiled into strands. Trypsin and thermolysin treatment removed the surface projections, and the G protein and high mol. wt. protein. Enzymically liberated G protein had a mol. wt. 5000 to 7000 smaller than attached G protein, suggesting that a 5000 to 7000 mol. wt. part of the G protein was left embedded in the membrane.

Lactoperoxidase- and chloramine T-catalysed iodination of intact SYVV particles labelled the G protein first, confirming its external location. The M1 and M2 proteins were the next labelled and were considered to be membrane associated. The N protein and the high mol. wt. protein were the last to be labelled.

INTRODUCTION

Sowthistle yellow vein virus (SYVV) is an enveloped bacilliform virus, transmitted in a persistent manner by the aphid Hyperomyzus lactucae L. and infecting sowthistle (Sonchus oleraceus L.) and lettuce (Lactuca sativa L.). In these plants infection causes vein clearing and vein banding symptoms (Duffus, 1963; Richardson & Sylvester, 1968; Peters, 1971). From the known morphological and physicochemical data, SYVV has been included in the rhabdovirus group.

Rhabdoviruses infecting plant and vertebrate cells differ in their morphology both in situ and in vitro and also in their cellular site of development and envelopment (Peters & Schultz, 1975). These differences warrant comparative studies of the structural proteins of rhabdoviruses infecting the two cell types.

The structural proteins of vesicular stomatitis virus (VSV), the type member of the rhabdovirus group, and rabies virus have been thoroughly studied (see review by Knudson, 1973). Details of the structural protein composition of rhabdoviruses infecting plants is

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limited to only two viruses, potato yellow dwarf virus (PYDV) which has four major structural proteins (Knudson & Macleod, 1972) and lettuce necrotic yellows virus (LNYV) which seems to have two major structural proteins (Francki & Randles, 1974). In a recent brief report, Schultz & Harrap (1976) demonstrated four major structural polypeptides in purified SYVV preparations.

Data on the location of the structural proteins of rhabdoviruses has likewise been obtained almost exclusively from viruses infecting vertebrates, notably VSV and rabies virus. It has been suggested that the G protein is associated with the surface projections, the N protein is the major nucleocapsid protein, the L and NS proteins are minor nucleocapsid proteins implicated in replicase activity and the M protein or proteins are associated with the virus membrane (Wagner et al. 1972; Knudson, 1973; Emerson & Yu, 1975; Imblum & Wagner, 1975). Little direct information is available for the location of the structural proteins of rhabdoviruses infecting plants.

In this communication we present some data concerning the characterization and location of the structural proteins of SYVV.

METHODS

Virus propagation and purification. SYVV was propagated in sowthistle (Sonchus oleraceus L.) plants grown under normal glasshouse conditions. The original virus isolate of Dr J. E. Duffus was kindly provided by him and used throughout. Aphids (Hyperomyzus lactucae L.) infected with SYVV were used to inoculate 4 to 5 week old plants. Infected leaves showing vein clearing symptoms (14 to 21 days p.i.) were used for virus purification. Virus was purified by a modified method of Peters & Kitajama (1970) as described by Ziemiecki & Peters (1976). The concentration of virus in purified preparations was determined in terms of protein as described by Lowry et al. (1951) using bovine serum albumin as a standard.

Electron microscopy. All samples were fixed with an equal volume of 2 % glutaraldehyde in double distilled water for 20 min prior to examination. Grids were floated on double distilled water for 1 min to remove excess glutaraldehyde, and stained with either 2 % phosphotungstic acid in double distilled water adjusted to pH 7.0 with NaOH, or with unbuffered 2 % uranyl acetate. Samples were examined in a Siemens Elmiskop 101 electron microscope.

Electrophoresis of protein. All samples for electrophoretic analysis were brought to 1 % of SDS and boiled for 5 min. Electrophoresis in cylindrical gels, staining and destaining were according to Weber & Osborne (1969). Protein from iodinated virus was also electrophoresed on a slab gel apparatus (Studier, 1973), using the discontinuous buffer system of Laemmli (1970), 3 % stacking gel and 11 % resolving gel. Electrophoresis was at 50 V for 5 to 8 h. Slab gels were stained with 0.125 % Coomassie brilliant blue G 250 (Merck) in 50 % methanol, 7 % acetic acid and destained in 50 % methanol, 7 % acetic acid. Stained gels were dried on to Whatman 3MM filter paper under vacuum, essentially as described by Maizel (1971). Prior to drying, the gels were soaked in a solution of 5 % glycerol, 50 % methanol for 1 h. Kodak medical X-ray film (RP Royal, X-Omat) was used to make autoradiograms.

Mol. wt. of the structural proteins were determined from a standard curve using the following proteins as markers: phosphorylase a (Sigma, 94000), bovine serum albumin (Sigma, 68000), catalase (Sigma, 60000), ovalbumin (Difco, 43000), pepsin (Sigma, 35000), concanavalin A (Sigma, 27000), papain (Sigma, 21000), trypsin (Sigma, 23000) and cytochrome c (Sigma, 11700). Glycoproteins were located following electrophoresis by the method of Zacharius, Zell & Morrison (1969).
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Detergent disruption. Purified virus was treated with saponin (Merck) at final concentrations varying between 0.05 to 1% in water, 0.1 M-tris/HCl buffer, pH 8.2, or 0.1 M-glycine, 0.1 M-magnesium acetate, pH 7.0 (GMA 2 buffer). Nonidet P40 (Shell Chemicals Ltd) was used at final concentrations varying between 0.05 to 1% in water, 0.1 M-tris/HCl, pH 8.2, 0.1 M-phosphate buffer, pH 7.6, or 0.1 M-glycine, 0.01 M-magnesium acetate, pH 7.0 (GMA 1 buffer). All treatments were at room temperature, the time of incubation varying between 1 and 60 min.

Enzyme treatment. The effects of the following enzymes on particle morphology and protein composition were investigated. The source, buffer used, temperature and time of incubation respectively are indicated in brackets. Trypsin (Sigma, 0.025 M-tris/HCl, pH 7.6, 37 °C, 0 to 3 h), thermolysin (Sigma, 0.1 M-tris/HCl, pH 7.6, containing 0.1 M-NaCl, 0.005 M-CaCl₂, 37 °C, 0 to 60 min), pronase (B grade, Calbiochem, 0.025 M-tris/HCl, pH 7.3, 37 °C, 0 to 60 min), bromelain (Sigma, 0.1 M-citrate, pH 4.5, or 0.1 M-tris/HCl, pH 7.2, 37 °C, 0 to 60 min). Enzyme-treated virus was examined in the electron microscope and, if desired, dissociated directly with SDS and electrophoresed. In some experiments using trypsin, and in all experiments using thermolysin, enzyme activity was inhibited prior to dissociation with SDS by soybean trypsin inhibitor (Sigma, final ratio of inhibitor to enzyme, 2:1) and EDTA (0.1 M-final concentration) respectively.

Iodination of intact and disrupted virus. Virus was disrupted by incubation with 0.1% Nonidet P40 at room temperature for 30 min prior to iodination. The reaction mixture for lactoperoxidase catalysed enzymic iodination was: 1.6 ml buffer (GMA 1 buffer, 1/10 strength GMA 1, or 0.05 M-tris/HCl, pH 7.5), containing 20 µl 0.1 M-potassium iodide, 50 µg intact or disrupted virus, 25 µl 0.25 mM-hydrogen peroxide (H₂O₂) and 25 µl carrier free ¹²⁵I, Amersham Radiochemical Centre, U.K.). The amount of ¹²⁵I used was varied depending on the ratio of ¹²⁵I to virus (µCi ¹²⁵I/µg SYVV = 0.001 to 0.33). The reaction was initiated by the addition of 25 µl lactoperoxidase solution (E₂₅₀ = 0.08) followed by gentle agitation, and continued by the addition of three 25 µl samples of 0.25 mM-H₂O₂ at 2 min intervals. The reaction was terminated when desired by the addition of 0.5 ml 0.01 M-cysteine hydrochloride and standing in ice. Intact labelled virus was collected by sedimenting through a 5% sucrose cushion (40000 rev/min in a SW 50.1 rotor). The pellet was resuspended in 0.1 ml 0.01 M-phosphate buffer, pH 7.0, containing 1% SDS and disrupted by boiling for 5 min.

Labelled virus disrupted by Nonidet P40 was precipitated with an equal volume of cold 25% trichloracetic acid, collected by centrifugation (10000 g for 10 min), and washed with cold ethanol. Excess ethanol was removed by evaporation and the proteins dissociated with SDS.

RESULTS

Structural proteins of SYVV

Four major and one minor proteins were observed following electrophoresis of purified virus on 5, 7.5 and 10% acrylamide gels. Incorporation of 2-mercaptoethanol into the disruption and electrophoresis buffers had no effect on the protein band patterns. Fig. 1, trace (a) shows the densitometer pattern of proteins from purified virus electrophoresed on a 7.5% acrylamide gel. The nomenclature adopted for the structural proteins is in accordance with the proposal of Wagner et al. (1972). The approximate mol. wt. of the structural proteins as calculated from 10% acrylamide gels were: 150000 (high mol. wt. protein), 83000 (G), 60000 (N), 44000 (M1), 36000 (M2). The amount of the minor high mol. wt. protein varied from preparation to preparation. The G protein sometimes appeared as two
adjacent resolvable bands (arrows, Fig. 1), the higher mol. wt. component having a mol. wt. of about 90000. This observation was not affected by the presence or absence of 2-mercaptoethanol in the disruption buffer.

As judged from periodic acid-Schiff’s staining, the 150000 mol. wt. component and G protein contained covalently bound carbohydrate (Fig. 1, trace b). Whenever the G protein appeared as two adjacent bands, both stained positive with periodic acid-Schiff’s reagent; however, it was not possible to detect any quantitative difference in the intensity of staining. Further evidence for the glycoprotein nature of the high mol. wt. component and G protein was obtained from mol. wt. determinations on different percentage acrylamide gels (Segrest & Jackson, 1972). The observed mol. wt. of these proteins decreased with increase in the percentage acrylamide of the gels (5, 7.5 and 10%) whereas the values for the other proteins remained constant.

Location of the structural proteins of SYVV

Three experimental approaches were employed: (1) the use of proteolytic enzymes to remove proteins external to the virus membrane; (2) the use of detergents to prepare subviral structures, and determination of the structural proteins present in such structures; (3) enzymic and non-enzymic iodination of purified virus preparations. The first two approaches were made in conjunction with electron microscopy.

Effects of enzymes on SYVV

Of the enzymes tried, only trypsin and thermolysin appeared to have any effect on virus particles. Fig. 2 and 3 show the effect on the particle morphology of treating SYVV preparations with trypsin (virus to enzyme ratio, 10:1, w/w, 37 °C, 1 h) and thermolysin (virus
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Fig. 2. Electron micrographs of untreated (a) and trypsin-treated (b, c, d) purified SYVV. For experimental conditions see text. (e) Shows a particle unpenetrated by stain and with no projections.

Fig. 3. Electron micrographs of untreated (a) and thermolysin-treated (b to f) purified SYVV. For experimental conditions see text.

to enzyme ratio, 5:1, w/w, 37 °C, 1 h) respectively. Enzyme treatment resulted in removal of the surface projections. Some damage to the membrane was evident; however, the enzymes did not penetrate the membrane appreciably as seen from the electrophoretic patterns (see below).

The effect of trypsin treatment on the structural protein profile is shown in Fig. 4. Trypsin-treated virus was pelleted through a 3 ml 5% sucrose cushion, dissociated with SDS and the
Fig. 4. Densitometer traces of Coomassie brilliant blue stained proteins of untreated SYVV (a), trypsin-treated SYVV (b), and Nonidet P40 disrupted, trypsin-treated virus (c). Approx. 30 µg of virus was used for each experiment. For details see text. The large peak arrowed in trace (c) is due to trypsin inhibitor protein, the small peak to the left of this represents trypsin and the peak to the right probably represents degradation products. The peak intermediate in position between G and N (trace c) is undegraded trypsin-cleaved G protein. Electrophoresis from left to right.

The electrophoretic patterns obtained following treatment of virus with trypsin for different time intervals is shown in Fig. 5. The reaction was stopped by bringing the reaction mixture to 1% of SDS and boiling immediately. The total disrupted mixture was applied on to the gels. There was a rapid decrease (approx. 5 to 7000) in the observed mol. wt. of the G protein (gels 1 and 2), and this was followed by a gradual decrease in the amount of the lower mol. wt. G protein. After 15 min treatment with trypsin all the projections and all the G protein were completely removed from intact virus, as judged from electron microscopy (Fig. 2) and analysis of structural proteins (Fig. 4 and 5). Thus the lower mol. wt. G protein observed on the gels in Fig. 5 (gels 2 to 4) represents that which has already been removed from the particles by trypsin action.

Treatment of intact virus with thermolysin for different times gave similar results (Fig. 6). In this case the reaction was stopped by the addition of EDTA prior to disruption with SDS.
Fig. 5 Polyacrylamide gels (7.5 %) of polypeptides from SYVV incubated with trypsin for different time intervals. Gels 1, 2, 3, 4 and 5 are for time 0 min, 15 min, 30 min, 60 min and 4 h respectively. Reaction was stopped by adjusting the whole mixture to 1 % SDS and boiling immediately. The entire SDS-dissociated reaction mixture was applied to the gels. Approx. 30 μg of virus was used for each gel. Electrophoresis is from top to bottom. The position of the high mol. wt. protein is indicated by an arrow.

Fig. 6 Polyacrylamide gels (7.5%) of polypeptides from SYVV incubated for different time intervals with thermolysin. Gels 1, 2, 3, 4 and 5 are for 0 min, 5 min, 15 min, 30 min and 60 min respectively. The reaction was stopped by the addition of EDTA. The entire SDS-dissociated mixture was applied to the gels. Approx. 30 μg of virus was used for each gel. Electrophoresis is from top to bottom. The position of the high mol. wt. protein is indicated by an arrow.

Fig. 7 Polyacrylamide gels (7.5 %) of polypeptides from SYVV incubated for different time intervals with trypsin. Gels 1, 2, 3 and 4 are for time 0 min, 15 min, 30 min and 60 min respectively. After electrophoresis the gels were stained for carbohydrate using the periodic acid-Schiff’s method. Approx. 50 μg SYVV was used per sample. Electrophoresis is from top to bottom.
Failure to inhibit thermolysin activity resulted in total digestion of the structural proteins during the SDS disruption procedure. Again, the entire SDS-dissociated mixture was applied on to the gels.

Under the experimental conditions used, the action of thermolysin was slower than that of trypsin. A gradual conversion of G protein to the lower mol. wt. form was seen (Fig. 6, gels 2 to 5), the decrease in mol. wt. being approx. 5 to 7000. The high mol. wt. protein (arrowed) was also slowly degraded. The apparent increase in the amount of protein M2 is an artifact explained by the similarity in mobilities of M2 and thermolysin. The membrane remained intact during thermolysin treatment, because all the structural proteins in Nonidet P40-disrupted virus were susceptible to thermolysin digestion.

The decrease in mol. wt. of the G protein observed following treatment with either enzyme was not due to loss of the carbohydrate moiety (Fig. 7). The carbohydrate remained associated with the lower mol. wt. G protein (Fig. 7, gels 2 to 4).

**Effect of detergents on SYVV**

Saponin treatment (1 % solution in GMA 2 buffer, 20 min at room temperature) did not completely remove the membrane and projections as residual membrane was visible adhering to the nucleocapsids (Fig. 8a, b, arrowed). However, under the experimental conditions employed, removal of the membrane resulted in the uncoiling of the bullet-shaped nucleocapsids into strands (Fig. 8d).
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Treatment with Nonidet P40 (0.2% solution in 0.1 M-phosphate buffer, pH 7.6) resulted in complete removal of the membrane and projections (Fig. 9); however, as in the case of saponin, under the experimental conditions employed, removal of the membrane resulted in breakdown of the bullet-shaped nucleocapsids into strands. For both detergents, the longer the period of incubation the more extensive the degree of degradation into strands, degradation occurring from either end of the nucleocapsid.

Detergent treatment yielded samples showing good detail of nucleocapsid structure (Fig. 8 and 9). The nucleocapsids were bullet-shaped with regular cross-striations, the striations at the hemispherical end being radial. In cross-section the nucleocapsids were spherical, hollow and frequently exhibited striations across and at right angles to the line of the circumference (Fig. 9d). Uncoiled portions of the nucleocapsids consisted of a continuous strand bearing regularly spaced disc-like subunits.

Attempts were made to isolate stable subviral structures following detergent treatment, by centrifugation on sucrose gradients. The G protein and the high mol. wt. protein were associated with the membrane at the top of the gradients and the N protein was found throughout the gradient associated with strands of various lengths. Bullet-shaped nucleocapsid structures were occasionally observed within the gradient. Proteins M1 and M2 could not be located unequivocally in any of the gradient fractions. This may be attributable to a
specific proteolytic activity recently demonstrated in purified SYVV preparations, which selectively degraded proteins M1 and M2 (Ziemiecki & Peters, 1976).

**Iodination of SYVV**

Purified virus preparations were iodinated using both the lactoperoxidase and the chloramine T catalysed methods. Fig. 10 shows an autoradiogram of an SDS-slab gel; samples 1 to 4 were catalysed by chloramine T (chloramine T to virus ratio, 1:50, 50 μg SYVV, 10 μCi 125I, the reaction being terminated by the addition of sodium metabisulphite after 10 s, 30 s, 1 min and 2 min, respectively. Samples 5 to 8 were catalysed by lactoperoxidase (50 μg SYVV), 10 μCi 125I, the reaction being stopped by the addition of cysteine hydrochloride after 30 s, 1 min, 3 min and 5 min, respectively.

Both reactions gave similar results. The G protein was clearly the first to become iodinated, label being detected after the shortest period of iodination (Fig. 10, lanes 1 and 5). The next proteins to be labelled were the M1 and M2 proteins (Fig. 10, lanes 3, 4, 6, 7) closely followed by the N protein (Fig. 10, lanes 4, 7, 8). Selective labelling of either the M1 or M2 proteins could not be demonstrated, both appearing to be labelled at approximately the same time. The high mol. wt. species (arrowed) was labelled late, at approximately the same time as the N protein. All the structural proteins were susceptible to labelling when Nonidet P40 disrupted virus was used; however, the N protein appeared to be labelled non-stoichiometrically, since there was less incorporation than expected. This may reflect unavailability of tyrosine residues due to N protein–RNA interactions or to relatively few tyrosine residues in N protein molecules being iodinatable.

**DISCUSSION**

The nomenclature adopted for the proteins of SYVV (Fig. 1) is according to the proposal of Wagner et al. (1972); however, this relies on location and function as criteria. The difficulty in ascertaining definitely the location and function of the proteins of SYVV (parti-
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icularly M1 and M2) may necessitate change in the nomenclature used here when more data become available.

The mol. wt. values obtained for the structural proteins in this study are similar to those quoted by Schultz & Harrap (1976), with the exception of G protein which they found to be 71,000. Subsequent determinations done in this laboratory using the same isolate of SYVV gave values similar to those reported here (M. G. Schultz, personal communication). The difference may reflect differences either in the conditions used to propagate the virus or in choice of protein markers and gel system.

Rhabdoviruses may be grouped into VSV-like and rabies-like viruses according to the protein patterns on SDS-gels (Lenoir & De Kinkelin, 1975). The electrophoretic patterns observed with purified SYVV put this virus in the rabies-like group, in contrast to PYDV whose protein profile is VSV-like (Knudson & MacLeod, 1972; Wagner, Schnaitman & Snyder, 1969). Any bearing of such a classification on morphology and biological properties remains to be investigated.

The G protein occasionally appeared as two adjacent bands, both containing covalently bound carbohydrate. This phenomenon has been observed previously with rabies virus (Sokol, Stancek & Koprowski, 1971), and several rhabdoviruses infecting fish (Hill et al. 1975). This may be due to different extents of glycosylation of the two species or to the existence of two distinct G protein polypeptides differing little in size, as in the case of the projection protein of Sindbis virus (Schlesinger, Schlesinger & Burge, 1972) and Semliki Forest virus (Simons, Keränen & Kääriäinen, 1973).

The situation regarding the high mol. wt. protein detected in purified SYVV preparations requires further investigation. A true L protein, defined as a distinct polypeptide coded for by the virus genome, associated with the nucleocapsid and functional in replicase activity (Stampfer & Baltimore, 1973; Emerson & Yu, 1975; Imblum & Wagner, 1975) may be present in small amounts; however, the data obtained on the SYVV high mol. wt. protein are not consistent with such a function for this protein. In our opinion this protein is probably a dimer of G protein. Its approximate mol. wt., the presence of carbohydrate, its removal by treatment with proteolytic enzymes and its association with the membrane fraction following Nonidet P40 treatment support our opinion. Sokol et al. (1971) observed on SDS-gels a 160,000 minor component following SDS/2 mercaptoethanol disruption of 3H-glucosamine labelled virus, which they suggested was a dimer of G protein. The L protein reported in association with purified envelopes from saponin treated VSV (Arstila, 1974) may be analogous to the high mol. wt. protein detected with SYVV. Stable aggregates of glycoproteins formed after SDS disruption have been observed previously (Tuech & Morrison, 1974). The observation, on SDS-gels, of large proteins distinct from the L protein should be borne in mind when naming rhabdovirus structural proteins.

Proteolytic enzyme treatment showed the G protein was associated with the surface projections (Fig. 2, 3, 4). Furthermore, the decrease in mol. wt. of the G protein resulting from enzymic removal of the surface projections suggests that a 5 to 7000 portion of the G protein penetrates into the membrane and is inaccessible to proteolytic action. The faint band seen at the electrophoretic front in Fig. 5 (gels 2 to 5) and Fig. 6 (gels 4 and 5) may represent this component. Similar sized hydrophobic fragments of the projection protein embedded in the membrane have been observed with Semliki Forest virus (Gahmberg, Utermann & Simons, 1972) and recently with VSV (Mudd, 1974; Schloemer & Wagner, 1975), and probably represent a characteristic of projection/membrane interaction.

Isolation of intact nucleocapsids was attempted in order to determine which structural proteins are present in intact nucleocapsids, thus testing the hypothesis that a protein or
proteins additional to the N protein is necessary to maintain the bullet-shaped nucleocapsid intact (Cartwright, Smale & Brown, 1970). However, these attempts using the detergents saponin and Nonidet P40 proved unsuccessful.

Electron microscopic observation of the structures resulting from detergent treatment (Fig. 8 and 9) supported the idea that the nucleocapsid of SYVV is a hollow tubular structure with one closed end, composed of a strand of nucleic acid bearing disc-like subunits of N protein. The strand is held together in the characteristic bullet-shaped structure by interactions between the N protein subunits and perhaps other protein(s) (Peters & Schultz, 1975).

Lactoperoxidase catalysed iodination has been shown to label specifically proteins on the external surface of biological membranes (Phillips & Morrison, 1970, 1971). This technique has been used to locate such proteins on several enveloped viruses (Stanley & Haslam, 1971; Walter & Mudd, 1973; Moore, Kelley & Wagner, 1974). In the present experiments, all the proteins could be labelled if the reaction was permitted to go on for a sufficiently long period (Fig. 10, lane 8). Labelling of proteins located on the inside of membranes, using this technique, has been observed (Walter & Mudd, 1973; Tsai, Huang & Canellakis, 1973) so that caution should be exercised in the interpretation of results obtained using enzyme catalysed iodination. As an additional check on the penetration of the membrane by the iodine, in the experiments described here, the course of iodination was followed on a time dependent basis (Fig. 10).

Iodination of intact virus particles confirmed the external location of the G protein, whereas proteins M1 and M2 are equally accessible to iodination and seem spatially intermediate between the projection and the nucleocapsid. This would suggest an association with the membrane, and hence the nomenclature adopted for these two proteins. The late labelling of the N protein is in agreement with its internal location. The late labelling of the high mol. wt. protein suggested an internal location; however, it did not preclude the possibility that it may be a dimer of G protein, a certain amount of iodination of G protein being needed before the high mol. wt. protein appeared labelled.

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