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Temperature-Sensitive Mutants of Vesicular Stomatitis Virus: Synthesis of Virus-Specific Proteins

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Viral proteins synthesized in L cells infected with temperature-sensitive (*ts*) mutants of vesicular stomatitis (VS) virus at permissive (31 C) and nonpermissive (39 C) temperatures were compared by polyacrylamide gel electrophoresis. Mutant *ts* 5, deficient in synthesis of viral ribonucleic acid (RNA^-), failed to synthesize any of the five identifiable viral proteins at 39 C. Each of three RNA^+ mutants, representing three separate complementation groups, showed distinctive patterns of viral protein synthesis at nonpermissive temperature. Equivalent amounts of ^3H-amino acids were incorporated into the five viral proteins made in cells infected with RNA^+ mutant *ts* 45 at 31 and 39 C. Complete virions of *ts* 45 could be identified by electron microscopy of infected cells incubated at the nonpermissive temperature; the defect in *ts* 45 appeared to be due in part to greater thermolability of virions as compared with the wild-type. RNA^+ mutant *ts* 23 was deficient in synthesis of viral envelope protein S and failed to make detectable virions at the nonpermissive temperature. Infection of cells at 39 C with the third RNA^+ mutant, *ts* 52, resulted in synthesis of all five viral proteins, but the peak of radioactivity representing the viral membrane glycoprotein migrated more rapidly on gels than coelectrophoresed authentic virion ^{14}C-glycoprotein or viral ^3H-glycoprotein extracted from cells infected at 31 C. These data and results of experiments on incorporation of radioactive glucosamine suggest that the primary defect in mutant *ts* 52 at nonpermissive temperature is failure of glycosylation of the viral glycoprotein. The viral structural proteins made in cells infected with *ts* 52 at the nonpermissive temperature did not assemble into sedimentable components as they did at permissive temperature; this observation indicates failure of insertion of the nonglycosylated protein (G′) into cell membrane. In support of this hypothesis was the finding that antiviral-antiferritin hybrid antibody did not detect VS viral antigen on the plasma membrane of L cells infected at 39 C with *ts* 52. In contrast, VS viral antigen localized in plasma membrane of L cells infected at 39 C with mutants *ts* 23 and *ts* 45 was readily detected by electron microscopy and fluorescence microscopy.

Conditional lethal mutants of vesicular stomatitis (VS) virus should provide incisive probes for analyzing synthesis and assembly of viral proteins as well as for other biochemical studies. Temperature-sensitive (*ts*) mutants of VS virus arise frequently upon induction with mutagenic agents (18) or spontaneously (6). All of the mutants described thus far exhibit relatively low rates of reversion and can be grown to high titer at permissive temperatures of 30 to 31 C. Growth of mutant viruses is markedly reduced at restrictive temperatures of 39 to 40 C, which fully support the wild type. Pringle (18) reported four complementation groups among his 175 mutagen-induced *ts* mutants of VS virus; Flamand (6) provided evidence that her 71 spontaneous *ts* mutants fall into five complementation groups. Reciprocal complementation studies reveal four complementation groups in common among the Glasgow and Orsay mutants (Flamand and Pringle, J. Gen. Virol., *in press* and C. R. Pringle, *personal communication*). Representative mutants in three complementation groups synthesize ribonucleic acid (RNA) at the restrictive temperature in amounts equivalent to the wild type, or almost so, and are classified as RNA^+ mutants; mutants in the other two complementation groups (RNA^- mutants) show markedly reduced synthesis of viral RNA at 39.8 C (6, 12, 16).

The genetic evidence for the existence of five

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cistrons in the genome of VS virus is quite consistent with biochemical studies reported to date. Wagner et al. (26) have identified by disc gel electrophoresis three structural and two nonstructural viral proteins in L cells infected with wild-type VS virus; the combined molecular weights of these five peptides account for about two-thirds of the coding potential of the VS virus genome (8, 26). The VS viral structural proteins are designated N for nucleoprotein, G for membrane glycoprotein, and S for membrane surface protein; the two nonstructural proteins are called NS1 and NS2. A minor peak that migrates slowly on gels (protein L) is probably an aggregate. We do not always detect the additional minor structural protein reported by Mudd and Summers (17) which migrates on their long gels approximately to the position of our protein NS1.

In this report we compare the structural VS viral proteins synthesized in L cells infected with wild-type and four *ts* mutant viruses, each representing a separate complementation group.

MATERIALS AND METHODS

The materials and many of the procedures were similar to those described in preceding papers (24–26).

Cells and media. Monolayer cultures of L cells and chick embryo (CE) fibroblasts were grown to confluency at 37 C in plastic petri plates (60 or 100 mm in diameter) or in plastic flasks (75-cm² surface) in a humidified atmosphere of ~5% CO_2 in air. Growth medium for CE cells consisted of 0.5% lactalbumin hydrolysate and 2% calf serum; medium 199 with 10% calf serum was used for growth of L cells. Media used for experimental infection were basal medium, Eagle's (BME) with or without leucine and tyrosine, depending on isotopes used for labeling. All media were purchased from Grand Island Biological Co., Grand Island, N.Y.

Viruses. The original wild type of Indiana serotype VS virus and the four *ts* mutants were kindly provided by A. Flamand and F. Lafay, Faculté des Sciences, Orsay, France. The pedigrees of these viruses have been described in detail (6). The temperature range was slightly modified from that used in France; our permissive temperature was 31 C and nonpermissive temperature was 39 C. Freeze-dried samples of the original *ts* mutants showed a slightly reduced titer and a somewhat greater proportion of revertants and leaky plaques. However, before all experiments, the *ts* mutants were cloned by plaque purification on L cells. Virus aspirated from a single isolated plaque was grown on CE cells at 31 C for 30 hr, and these stocks were stored at −60 C. All experimental cultures were incubated at temperatures controlled at 31 ± 0.5 or 39 ± 0.5 C in a humidified atmosphere of 5% CO_2 in air. L-cell monolayers were used for plaque assay of viruses adsorbed for 1 hr at 31 C and incubated at 31 C or 39 C for 48 to 72 hr when plaques were scored. Titers per milliliter of stock mutant viruses plated at 31 C ranged from 8×10^8 to 3×10^9 plaque-forming units (PFU).

Radioisotopes. ^{3}H-leucine (58 Ci/mmole) and ^{3}H-tyrosine (50 Ci/mmole) were obtained from Schwarz BioResearch, Orangeburg, N.Y. Reconstituted uniformly labeled ^{14}C-amino acids (52 mCi/matom) were obtained from Amersham/Searle, Chicago, Ill. D-Glucosamine-*6-*^{3}*H* (1.3 Ci/mmole) was obtained from New England Nuclear Corp., Boston, Mass.

Infection, ^{3}H-labeling, and protein extraction of L cells. Confluent monolayer cultures of L cells in plastic petri plates were first exposed for 1 hr at 37 C to 5 or 10 ml of medium 199 containing 0.5 μg of actinomycin D per ml. Drained cultures in small plates were then infected with 0.2 ml and those in large plates with 0.5 ml of virus at input multiplicities of 40 to 80 PFU; virus was allowed to adsorb for 40 min at 31 C. The cell layers in the small plates were covered with 1.5 ml and in the large plates with 5 ml of special BME devoid of leucine and tyrosine and free from serum. After 2 hr, a mixture of ^{3}H-leucine and ^{3}H-tyrosine was added to the medium to provide a concentration of 5 μCi of each isotope per ml. Cell monolayers to be harvested were drained free of medium, washed twice with large amounts of ice-cold phosphate-buffered saline, and drained again. The cells in each monolayer were then scraped with a rubber policeman into 0.4 ml of Earle's saline solution. Proteins were extracted from whole cells or from cytoplasmic contents of cells disrupted with a Dounce homogenizer in precisely the same way as proteins were extracted from purified VS virions (24). After adding 0.1 volume of glacial acetic acid, a 100-μliter suspension was made 0.5 M with respect to urea and 1% with respect to sodium dodecyl sulfate (SDS). After incubation for 1 hr at 37 C, the protein extract was dialyzed at room temperature for 16 to 18 hr against 250 ml of phosphate buffer (*p*H 7.2, 0.01 M) containing 0.1% SDS, 0.5 M urea, and 0.1% 2-mercaptoethanol.

Marker ^{14}C-proteins of VS virions. As previously described (24, 26), structural proteins extracted from purified virions were labeled with ^{14}C-amino acids (1.13 μCi/ml) to provide authentic VS viral protein markers for coelectrophoresis with the intracellular experimental viral ^{3}H-proteins. Released labeled virions were purified by differential and rate zonal centrifugation; their proteins were extracted with urea and SDS and stored at −60 C.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed as previously described (24, 26) by using 7.5% acrylamide gels at 5 ma/gel for 6.5 hr. Gels sliced into lengths of 1.25 mm were dissolved in 0.5 ml of Nuclear-Chicago solubilizer and diluted in toluene-based fluors; disintegrations of ^{3}H-protein extracted from infected cells and marker virion ^{14}C-proteins were counted by scintillation spectrometry assisted by a double-labeling computer program.

Electron microscopy. The presence of VS virus was monitored by electron microscopy of unfixed, whole, infected L cells negatively stained with phosphotungstic acid (PTA) or of fixed, sectioned cells positively stained with uranyl acetate and lead citrate. Preparation of specimens and their examination in a

Siemens Elmiskop 1A microscope have been described (7, 23, 25).

Ferritin-antibody staining. The technique for preparing hybrid 5*S* bivalent antibody in which one antigen-combining site is reactive with VS viral antigen and the other with ferritin has been reported (23). After this hybrid antibody had reacted specifically with viral antigen on the surface of unfixed cells, its presence was detected by exposing the cells to ferritin; the labeled cells were then fixed, stained, sectioned, and examined by electron microscopy. The hybrid antibody was also detected by exposing unfixed, infected cells to fluorescein-labeled apoferritin for examination by fluorescence microscopy. The same sera and reagents were used in these studies as those described in the paper on the methodology of this technique (23).

RESULTS

Characterization of the ts mutants. Owing to a certain degree of instability of VS viral *ts* mutants after prolonged storage or repeated passage (A. Flamand, *personal communication*), there is no assurance of strict identity of our mutant stocks compared with the original strains received from France. Nevertheless, we have used the nomenclature for the mutants reported by Flamand (6) on the basis that plaque-purified clones were prepared directly from her stocks. The infectivity of these clones and synthesis of viral RNA at nonpermissive temperature corresponded closely to the original Orsay mutants. Their relationship to the Glasgow complementation groups is summarized in Table 1.

Table 1 also shows the plating efficiency on L-cell monolayers of our cloned stocks of wild-type VS virus and each of four *ts* mutants at 31 and 39 C. In each case, decimally diluted virus was adsorbed on duplicate plates for 1 hr at 31 C and then incubated at 31 or 39 C. Plaques were scored at 72 hr but did not increase in number after incubation at 39 C for 48 hr. Plaque titers of the mutants at 31 C were only slightly lower than the titers of the wild type at 31 or 39 C. Table 1 also indicates which of the mutants synthesizes viral RNA at 39 C. Detailed analysis by M. P. Kiley of RNA synthesis in cells infected with each mutant is in progress, but preliminary results indicate that only *ts* 5 shows marked reduction of RNA synthesis at the restrictive temperature (20% or less of the RNA made at permissive temperature), whereas the wild type and the other three mutants make relatively normal amounts of RNA (70% or greater) at the restrictive temperature.

These results confirm the observations of the French workers (6, 12) and provide a reliable basis for comparing protein synthesis in cells infected with these *ts* mutants and the wild type from which they were derived.

TABLE 1. *Plating efficiency and capacity to synthesize viral RNA at nonpermissive temperature of wild-type vesicular stomatitis (VS) virus and ts mutants representing four complementation groups*

Virus	Group[a]	PFU/ml on L cells		RNA made at 39 C[b]
		31 C	39 C	
Wild type		3.7×10^9	3.4×10^9	+
ts 5	I	8.9×10^8	2.0×10^3	−
ts 23	III	2.0×10^9	6.0×10^3	+
ts 45	V	1.3×10^9	2.5×10^3	+
ts 52	II	3.1×10^9	2.5×10^3	+[c]

[a] This classification of the Orsay mutants into reciprocal complementation groups is based on the nomenclature for the Glasgow *ts* mutants of VS virus devised by Pringle (17) and reported jointly by Flamand and Pringle (J. Gen. Virol., *in press*). The Orsay mutant *ts* 45 complements with Glasgow groups I, II, III, and IV and is therefore designated group V. RNA$^-$ mutants representative of complementation group IV were not included in our studies.

[b] Comparison of acid-precipitable counts of ^{3}H-uridine incorporated in duplicate cultures treated with actinomycin, infected at a multiplicity of 50, and incubated at 31 or 39 C. RNA$^-$ signifies >80% reduction at nonpermissive temperature compared with <20% for RNA$^+$.

[c] Mutants in complementation group II tend to be genetically unstable and may sometimes be deficient at 39 C in RNA synthesis (Pringle, *personal communication*).

VS virus-specific proteins synthesized in L cells infected with ts mutants. Synthesis of VS viral proteins in cells infected with wild-type virus at 37 C begins at 1 to 2 hr after infection, reaches a peak by 3 to 4 hr, but continues beyond 5 hr (26). There appears to be no regulation in the order of synthesis of the five identifiable viral proteins, each of which is made throughout the cycle of infection, although in different amounts. The nucleoprotein (N) is synthesized in much larger amounts than the envelope proteins G and S; the nonstructural protein NS1 always exceeds NS2, which is sometimes difficult to detect. Protein L (for large) is always present, but its significance is unclear (24). At high multiplicities of infection, normal cellular protein synthesis is switched off by 2 hr (27). In most of these experiments with *ts* mutants, the period of labeling with ^{3}H-amino acids was chosen as 2 to 5 hr after infection, the time at which viral synthesis is most active in cells infected with the wild type. Shorter pulses of 1 hr each provided no significant advantage but

served to confirm with the mutants the relatively constant rate of viral protein synthesis from 2 to 5 hr after infection with wild-type virus.

Monolayer cultures of L cells were infected with wild-type VS virus or each of the 4 *ts* mutants. Duplicate plates were incubated at 31 or 39 C; ^{3}H-amino acids were added at 2 or 4 hr and the cells were harvested at 5 hr. Proteins were extracted from unfractionated infected cells and analyzed by electrophoresis on 7.5% neutral polyacrylamide gels containing urea and SDS. Marker ^{14}C-proteins extracted from purified virions were coelectrophoresed in every gel along with ^{3}H-proteins extracted from infected cells.

Figure 1 shows the electrophoretic profiles of the viral proteins extracted from L cells infected with each of the four *ts* mutants at permissive and nonpermissive temperatures. The results with wild-type virus are not shown, but it exhibited on repeated determinations perfectly normal synthesis of all five viral proteins at both 31 and 39 C as well as 37 C. No consistent differences could be detected in proteins synthesized at 31 C in cells infected with any of the four mutants; all proteins were present in approxi-

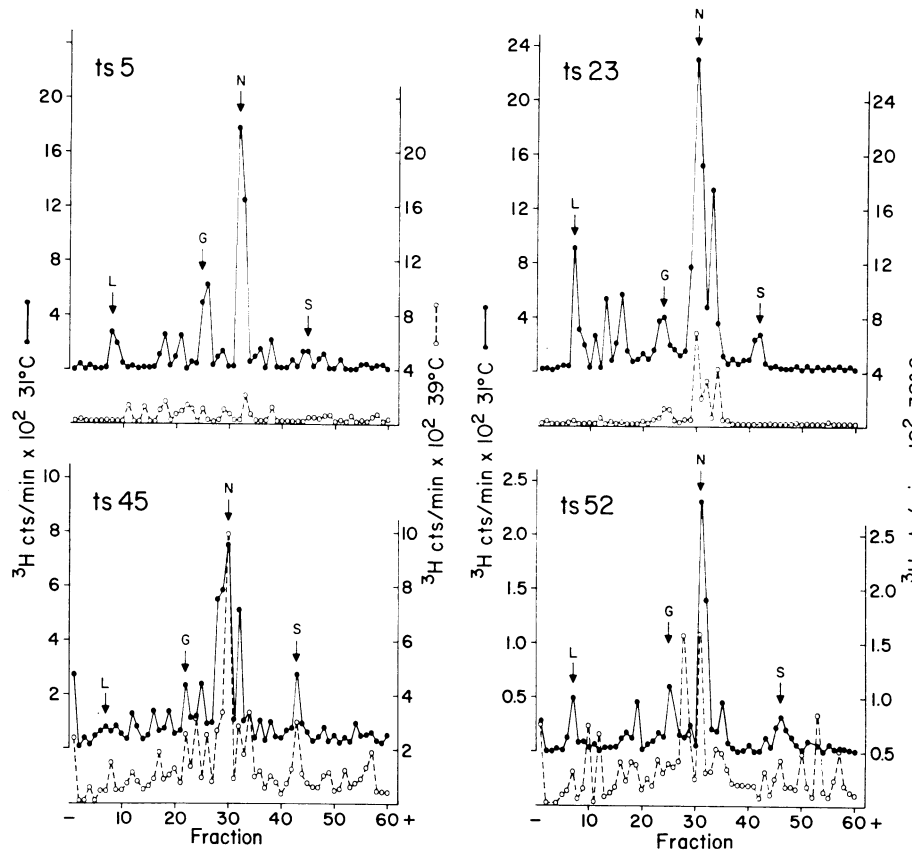

FIG. 1. *Comparative electropherograms of virus-specific proteins extracted from L cells infected at a multiplicity of ~ 50 with ts 5, ts 23, ts 45, or ts 52 at permissive (solid line) or nonpermissive (broken line) temperature. After an adsorption period of 40 min at 31 C, each plate (60 mm in diameter) was covered with 1.5 ml of special BME and incubated at 31 or 39 C. ^{3}H-leucine and ^{3}H-tyrosine (5 μCi/ml of each) were added to the cultures 2 hr after adsorption of ts 5, ts 23, and ts 45; cells were harvested after 3 hr of labeling. Cultures infected with ts 52 were labeled with the ^{3}H-amino acids for 1 hr (4 to 5 hr postinfection). Cells from each culture were washed with phosphate-buffered saline and scraped into 0.4 ml of Earle's saline solution. The proteins of each whole cell suspension were extracted sequentially with acetic acid, 0.5 M urea, 1% SDS, and 0.1% 2-mercaptoethanol. The extracted ^{3}H-proteins of each sample were subjected to electrophoresis on 7.5% neutral SDS acrylamide gels along with marker structural ^{14}C-proteins extracted from purified virions. The vertical arrows show the peak positions of the four marker ^{14}C-proteins. The variable peaks that migrated just distal to protein N represent NS1. NS2 can be seen as a small peak distal to protein S in some gels. The other slow-moving peaks represent either cellular proteins or aggregates of viral proteins (24–26).*

mately normal amounts except for the minor degrees of variability inherent in the system. In contrast, the RNA^- mutant *ts* 5 failed to synthesize appreciable amounts of any of the five viral proteins at 39 C. Parenthetically, the marked reduction of cellular protein synthesis in cells infected with *ts* 5 at the nonpermissive temperature provides additional evidence that switch-off of cellular protein synthesis does not require viral protein synthesis (27).

Among the RNA^+ mutants of different complementation groups, *ts* 45 showed no significant impairment of capacity to synthesize any of the five viral proteins at the restrictive temperature (Fig. 1). On the other hand, the RNA^+ mutant *ts* 23 showed reduced levels of synthesis of all proteins and failed to synthesize protein S at 39 C (Fig. 1). On repeated experiments, *ts* 23 synthesized far less protein S at nonpermissive temperature compared with that in duplicate cultures at permissive temperature; the relative proportion of the other viral proteins in cells infected with *ts* 23 were not significantly different at 31 and 39 C. The mutant *ts* 52 synthesized all viral proteins at the nonpermissive temperature but, quite strikingly, the ^3H-protein peak in the region of marker ^{14}C-protein G was absent or markedly diminished. Instead, however, a ^3H-protein peak that migrated faster than marker ^{14}C-protein G was present in cells infected with *ts* 52 at the restrictive temperature (Fig. 1). This mutant protein, hereby designated G′, should have an apparent molecular weight of approximately 75,000 daltons compared with 80,000 daltons previously estimated (24) for the authentic structural protein G extracted from the VS virion.

Defective glycosylation of mutant ts 52 glycoprotein. The preceding experiments raised the possibility that the lower molecular weight of the G′ protein of *ts* 52 made at 39 C could be due to failure of glycosylation at the nonpermissive temperature. Only the G protein of wild-type VS virus is specifically labeled with radioactive glucosamine and other sugar precursors to make a covalently bonded glycoprotein (2, 15, 26). Digestion of the purified viral glycoprotein with Pronase results in liberation of a relatively homogeneous glycopeptide, the molecular weight of which has been estimated by gel filtration as ~4,000 daltons (2, 15). Therefore, complete failure of glycosylation of the viral precursor glycoprotein G′ could explain its lower molecular weight as evidenced by more rapid migration on gels.

We encountered difficulties of two kinds in attempts to demonstrate differential incorporation of radioactive sugars into the intracellular viral glycoprotein of *ts* 52 at 31 and 39 C. First, infection with VS virus apparently does not switch off normal glycosylation of cell-specific glycoproteins and glycolipids as it does cellular protein synthesis (27); therefore, background radioactivity of whole infected cells, compared with released virus, was generally too high to detect differential labeling of the intracellular virus-specific glycoprotein. Second, prolonged periods of labeling with radioactive sugars often resulted in their conversion to radioactive amino acids which were incorporated indiscriminately into viral and cellular proteins. These technical obstacles were finally overcome by devising a special labeling medium consisting of BME completely devoid of glucose and serum but containing twice the usual concentration of all amino acids (Grand Island Biological Co.). Cells infected with wild-type VS virus or *ts* 52 were incubated in this medium for 5 hr and labeled with ^3H-glucosamine from 2 to 5 hr.

The electropherograms in Fig. 2 demonstrate that ^3H-glucosamine was specifically incorporated into the viral glycoprotein made in L cells infected with *ts* 52 at 31 C but not at 39 C. Some large molecular weight components, presumably cellular in origin, were noted at the top of the gels, but virtually no label was present in the region of proteins N and S made at either temperature. In striking contrast was the very high degree of incorporation at 39 C as well as 31 C of ^3H-glucosamine (or its derivatives) into components of low molecular weight which migrated on gels in a manner described for glycolipids (15, 19). This incorporation of sugar precursors into glycolipids is interpreted as evidence that the normal sugar transferases of infected L cells function efficiently at nonpermissive as well as permissive temperatures. It seems reasonable to assume, therefore, that failure of glycosylation of the *ts* 52 viral glycoprotein is not due to restriction at the cellular level but is an intrinsic defect of the viral precursor glycoprotein (G′) which fails to serve as a sugar acceptor, presumably owing to conformational changes at the nonpermissive temperature.

Sedimentable and nonsedimentable viral protein fractions of disrupted L cells infected with ts mutants. It was shown in a previous study (26) that intracellular VS viral proteins can be separated into soluble and insoluble components by high-speed centrifugation after homogenization of infected cells. The nonstructural proteins remain in the nonsedimentable fraction throughout the cycle of infection, whereas the structural N protein appears first as a soluble component and later aggregates into a particulate component. The G and S structural proteins of the virion envelope

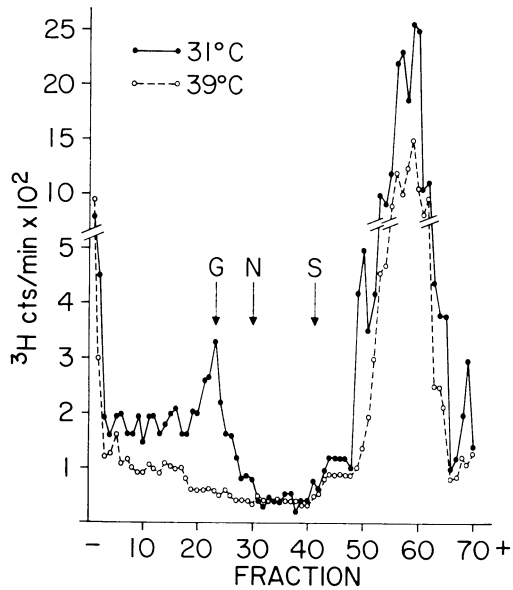

FIG. 2. *Differential incorporation of ^{3}H-glucosamine into virus-specific glycoprotein synthesized in L cells infected with mutant ts 52 at permissive (31 C) and nonpermissive (39 C) temperatures. Monolayer cultures of 2 × 10^7 L cells were infected with ts 52 at a multiplicity of 80; virus was adsorbed at 31 C for 40 min. Duplicate cultures were incubated at 31 or 39 C in special BME free of glucose and serum but containing twice the usual amounts of all amino acids. ^{3}H-glucosamine (4 μCi/ml) was added to each culture at 2 hr, and the cells were harvested 5 hr postinfection. Proteins were extracted from the cells with urea and SDS, boiled for 1 min, and electrophoresed on 7.5% acryladmide gels along with marker ^{14}C-proteins extracted from purified VS virions (arrows refer to peak migration of viral proteins G, N, and S). Note the very large amount of ^{3}H label in rapidly migrating and relatively heterogeneous components which presumably represent glycolipids (19).*

are found predominantly in the sedimentable fraction throughout infection. Moreover, the G and S proteins are readily solubilized by the membrane-solubilizing agent digitonin, whereas the particulate N protein is not (26). From these and other data we concluded, tentatively, that N protein complexes with viral RNA to form the viral nucleocapsid, whereas the G and S proteins are inserted directly into cellular membrane (presumably plasma membrane) to form the virion envelope.

It was of considerable interest, therefore, to examine the distribution of the individual viral proteins in cells infected with RNA$^+$ *ts* mutants at permissive and nonpermissive temperatures. The basic hypothesis tested by these experiments was that the defectiveness of the RNA$^+$ mutants is at least partially related to maturation arrest of the viral structural proteins synthesized at the restrictive temperature. To this end L cells were infected with RNA$^+$ *ts* mutants at high multiplicity, incubated at 31 or 39 C, and labeled with ^{3}H-amino acids from 2 to 5 hr. The harvested cells were disrupted in a Dounce homogenizer, the nuclei and other large particulate matter were removed by low-speed centrifugation, and this supernatant was separated into sedimentable and nonsedimentable fractions by high-speed centrifugation. Proteins were extracted from the reconstituted pellet and from the supernatant fraction and analyzed by gel electrophoresis (25, 26).

Mutants *ts* 23 and *ts* 45 closely resembled wild-type VS virus; after infection for 5 hr at 39 C as well as at 31 C, greater than 90% of proteins N, G, and S were present in the sedimentable fraction. Protein S was again greatly reduced in cells infected with *ts* 23 at 39 C, but most of what was present remained in the sedimentable fraction. Figure 3 reveals that strikingly different results were obtained with mutant *ts* 52. The structural proteins were found predominantly in the sedimentable fraction of cells infected at 31 C, whereas the nonstructural proteins were almost entirely in the nonsedimentable fraction. However, even at permissive temperature there was somewhat more protein G and S in the nonsedimentable fraction than there was in cells infected with the wild type, *ts* 23, or *ts* 45. In cells infected with *ts* 52 at the restrictive temperature of 39 C, all of the viral proteins, structural as well as nonstructural, were recovered largely in the nonsedimentable fraction (Fig. 3). Only protein S was present in significant amounts in the sedimentable fraction. Once again, at 39 C a soluble protein peak was present distal to the ^{14}C-protein G marker, equivalent to the relative migration of the G′ protein of *ts* 52 at 39 C shown in Fig. 1.

These data are consistent with the hypothesis that defective glycosylation of mutant *ts* 52 at restrictive temperature results secondarily in failure of insertion of viral protein G into cell membrane which, in turn, leads to a block in assembly of other virion proteins.

Electron microscopy of L cells infected with ts mutants. There are many possible explanations for the low yield of infectious virus from cells infected with *ts* mutants at nonpermissive temperature. In the case of the RNA$^-$ *ts* 5 mutant, markedly reduced synthesis of viral RNA and viral protein is sufficient reason for low yield of infectious virus and, presumably, for defective virus as well. Among the possible explanations for abortive infection by RNA$^+$ *ts* mutants, which synthesize some or all of the viral proteins at

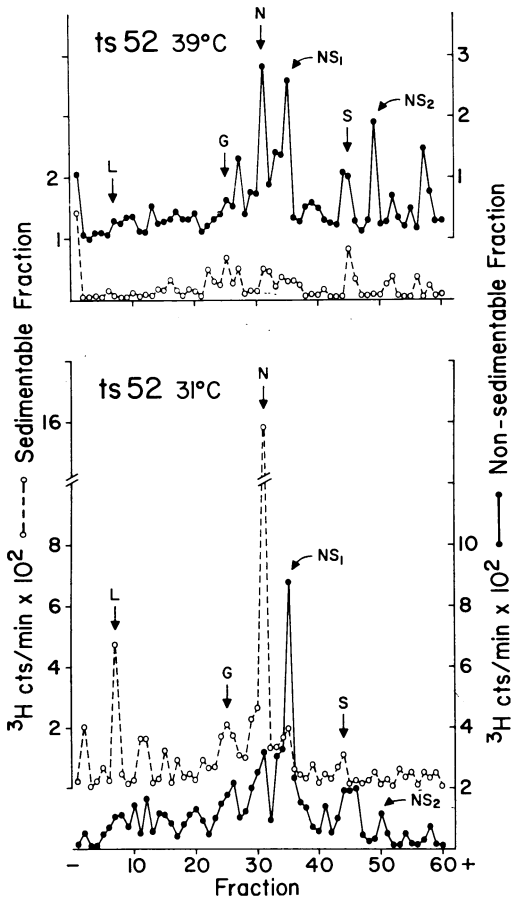

FIG. 3. *Comparative amounts of VS viral ^{3}H-proteins associated with nonsedimentable (solid lines) and sedimentable (broken lines) fractions of L cells infected with ts 52 at 31 and 39 C at a multiplicity of ~50. After an adsorption period of 40 min at 31 C, cell monolayers in petri plates (100 mm in diameter) were covered with 5 ml of special BME and incubated at 31 or 39 C. ^{3}H-leucine and ^{3}H-tyrosine (5 μCi/ml of each) were added to the cultures at 2 hr after adsorption and the cells were harvested at 5 hr (3-hr labeling time). Cells of each culture (~2 × 10^7 cells) were washed with phosphate-buffered saline and then scraped into 2 ml of Earle's saline solution. The cells were pelleted at 900 × g, resuspended in 0.8 ml of 10^{-2} M ethylene-diaminetetraacetic acid, 2 × 10^{-2} M tris (hydroxymethyl) aminomethane buffer (pH 7.2) for 20 min at 0 C, and disrupted by 20 strokes of a tight-fitting Dounce homogenizer. The nuclei and gross cellular debris were removed by centrifugation at 900 × g for 10 min. The cytoplasmic contents were then separated into nonsedimentable and sedimentable fractions by centrifugation at 130,000 × g for 90 min. Proteins were extracted from the supernatant fluid and reconstituted pellet and analyzed on 7.5% neutral SDS acrylamide gels along with virion marker ^{14}C-proteins (L, G, N, and S). Also shown are the positions of two nonstructural viral proteins, NS1 and NS2.*

nonpermissive temperature, are: (i) defective assembly, as appears to be the case with *ts* 52; (ii) assembly into defective, noninfectious virions, such as T particles (9) of VS virus; or (iii) complete assembly of B particles that are otherwise defective.

These alternative explanations for low infectivity of RNA$^+$ *ts* mutants were examined by electron microscopy of infected L cells scraped from monolayer cultures and negatively stained with PTA or fixed in glutaraldehyde and OsO_4, embedded in Epon 812, and stained with uranyl acetate and lead citrate. Representative examples of cells incubated at 31 C for 8 hr after infection, in this case with *ts* 52, are shown in Fig. 4 to demonstrate the appearance of released virions negatively stained with PTA and in Fig. 5A to demonstrate sectioned budding virions. Note the predominance of the B form of VS virions.

Table 2 summarizes the results of three separate experiments designed to demonstrate the presence or absence of characteristic VS virions in preparations of negatively stained or sectioned L cells infected for 8 hr at 31 or 39 C with wild-type virus or each RNA$^+$ *ts* mutant. No attempt was made to quantitate the numbers of virions, but the results are scored plus or minus after examining about 100 cells. The wild type and all four mutants produced abundant virions, predominantly B particles, at 31 C. Equivalent numbers of B virions were produced by infection with wild-type virus at 39 C; cells infected with the RNA$^-$ mutant *ts* 5 (not shown in Table 2) produced virions only at 31 C. Among the RNA$^+$ mutants representing three different complementation groups, *ts* 23 and *ts* 52 produced no virions detectable by electron microscopy of either negatively stained or sectioned preparations of L cells infected at 39 C. In contrast, virions were readily detected in preparations of cells infected with *ts* 45 at the nonpermissive as well as the permissive temperature. These virions were predominantly of the B type; therefore, their defectiveness must be due to reasons other than formation of truncated T virions at 39 C.

Thermolability of mutant ts 45. Preliminary data on the reason for defectiveness of *ts* 45 at 39 C were sought by comparing thermal inactivation of wild-type virus and *ts* mutants plated on monolayers of L cells (12, 13). After incubation in a water bath at 39 C, the plaque titer of the wild type did not diminish at 90 or 180 min. On the other hand, the infectivity of *ts* 23 fell by 60% and that of *ts* 45 by 83% in 3 hr. These data suggest that a partial explanation for reduction in plating efficiency of *ts* 45 virus at 39 C (see Table 1) is inactivation of virions at this tem-

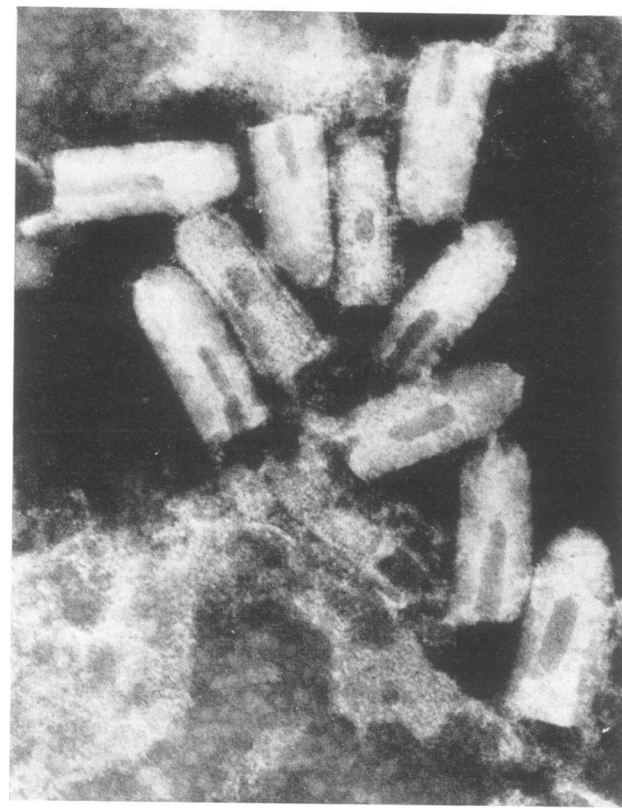

FIG. 4. *Electron micrograph of unfixed L cells infected with VS virus mutant ts 52 at 31 C for 8 hr and negatively stained with PTA.* × *140,000.*

perature rather than deficiency in synthesis or assembly of proteins.

Insertion of viral antigen into plasma membrane of L cells infected with ts mutants. The finding that structural proteins made in L cells infected with *ts* 52 at 39 C are nonsedimentable suggests that the envelope proteins of *ts* 52, in contrast to those of *ts* 23 and *ts* 45, are not inserted into plasma membrane. If this hypothesis is correct, viral antigen should not be detectable in the plasma membrane of cells infected with *ts* 52 at 39 C. As reported previously (23), VS viral antigen can be located selectively on the surface of infected cells by exposing them to antiviral-antiferritin hybrid antibody and then to ferritin, which is detected by electron microscopy, or to fluorescein-conjugated apoferritin, which is detected by fluorescence microscopy.

Monolayer cultures of L cells grown on cover slips were infected at multiplicities of 40 to 80 PFU/cell with wild-type virus or RNA^+ *ts* mutants. After incubation for 8 hr at 31 or 39 C, the infected cells were washed, exposed to antiviral-antiferritin hybrid antibody, washed again, scraped into test tubes, exposed to ferritin, washed again, and examined, as previously described (23), by electron microscopy and fluorescence microscopy. All specimens were coded, read as a double-blind experiment along with appropriate controls lacking virus or antibody, and scored as 0 to 4+ ferritin labeling.

The representative electron micrographs shown in Fig. 5 demonstrate almost complete absence of ferritin labeling of the plasma membrane of L cells infected with *ts* 52 at 39 C compared with the presence of abundant electron-dense ferritin on plasma membrane and budding virions from a culture incubated at 31 C. These data were confirmed by independent fluorescence microscopy of samples of the same infected cells.

Table 2 summarizes the data from two separate experiments in which cells infected at 31 and 39 C with wild-type virus or the RNA^+ mutants were compared on the basis of ferritin antibody labeling of plasma membrane. As noted, only plasma membrane of cells infected with *ts* 52 at 39 C and exposed to hybrid antibody was not

labeled with ferritin (as determined both by electron and fluorescence microscopy). Viral antigen was readily detected on the surface of cells infected with *ts* 52 at 31 C and all other infected cells at either temperature. It is of some interest, as noted in Table 2, that at nonpermissive temperature *ts* 45 produced both membrane antigen and progeny virions and *ts* 23

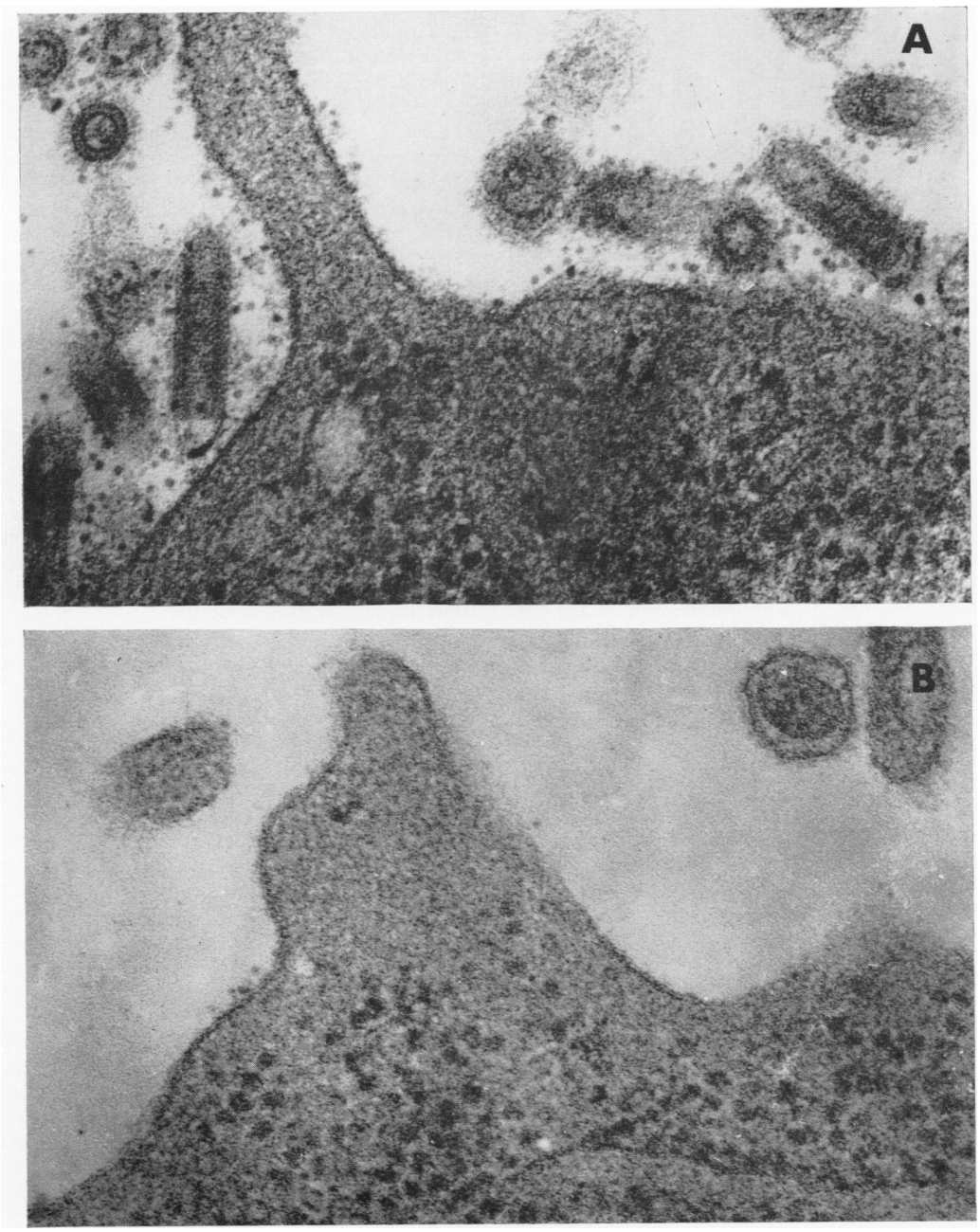

FIG. 5. *Electron micrographs of L cells infected with VS virus mutant ts 52 and incubated for 8 hr at 31 C (A) or 39 C (B). Each preparation of cells was exposed to antiviral-antiferritin hybrid antibody and then to ferritin before fixing; sectioning and staining are as described in the text (23).* × *140,000.*

TABLE 2. *Presence or absence of virions and viral antigen on the plasma membrane of L cells infected wtih wild-type vesicular stomatitis (VS) virus and RNA$^+$ ts mutants*

Virus	Virions[a]		Relative ferritin surface staining[b]	
	31 C	39 C	31 C	39 C
Uninfected		−		−
Wild type	+	+	+++	++++
ts 23	+	−	+++	+++
ts 45	+	+	++++	++
ts 52	+	−	++++	−

[a] Presence of virions in association with infected cells was determined by phosphotungstic acid negative staining (Fig. 4), as well as by thin sections of fixed and stained cells (Fig. 5). Cells were examined 8 hr after infection.

[b] L cells grown on cover slips in petri plates were infected with each virus at a multiplicity of ~80; viruses were allowed to adsorb for 40 min at 31 C before the cell layers were covered with 5 ml of Eagle's basal medium containing 2% calf serum. After incubation at 31 or 39 C for 8 hr, the cell layers were washed, exposed to antiviral-antiferritin hybrid antibody, washed again, and scraped into test tubes. Pelleted duplicate samples of cells were exposed to ferritin for electron microscopy or to fluorescein-conjugated apoferritin for fluorescence microscopy. Each specimen was scored independently by four investigators. Representative results of electron microscopy of cells infected with *ts* 52 at 31 and 39 C are shown in Fig. 5.

produced only membrane antigen, whereas *ts* 52 produced neither virions nor membrane antigen.

DISCUSSION

These experiments indicate that VS virus can serve as an important model for correlating the genetics and physiology of enveloped viruses that contain RNA. The recognition thus far of only five complementation groups (6) and five viral proteins (26) provides the basis for identifying the product of each cistron, a feat which has not been accomplished definitively with an animal virus. Mapping the genes of VS virus poses a much greater problem, but Pringle (18) has provided evidence for viral recombination. However, it should be noted also that the recognized proteins synthesized by VS virus account for only two-thirds of the potential genetic information of VS virus (26). This estimate holds, even if one includes the presumptive fourth minor structural protein described by Mudd and Summers (17) or if one considers the roughly 10 to 20% discrepancies in molecular weights of the major VS viral structural proteins reported by different laboratories (2, 5, 11, 17, 24). It can be assumed that one cistron of the VS virus genome codes for the RNA transcriptase found in purified virions (1), which could conceivably represent the minor fourth structural protein (17). The isolation of RNA$^-$ mutants in two separate complementation groups (6) suggests the existence of another cistron, the product of which should be a replicase concerned with synthesis of progeny RNA. We have made no attempt thus far to determine the functions of the two nonstructural proteins (NS1 and NS2) found in cells infected with VS virus, but it would seem logical to postulate that they are enzymes concerned with viral RNA synthesis.

Our studies of the RNA$^-$ mutant *ts* 5 strongly suggest that viral RNA synthesis is required for synthesis of viral nonstructural and structural proteins. A defective virion transcriptase that does not function at the nonpermissive temperature could explain the restriction at 39 C of both RNA and protein synthesis in cells infected with *ts* 5. If this model is correct, the mutants representing the other RNA$^-$ complementation group should synthesize viral messengers (8) and perhaps proteins but could be blocked in production of progeny RNA. Mutants of this class (Glasgow group IV) have not yet been examined.

The present studies with the RNA$^+$ mutants of VS virus provide evidence for identification of the gene products for two of the three known complementation groups in this class, both concerned with structural proteins of the virion envelope. Very little can be said about the thermolabile mutant *ts* 45; its increased rate of inactivation at 39 C could, of course, be due to a lesion in any of the three (or four) structural proteins. The defect in *ts* 45 is not likely to be in the virion replicase because cells infected at nonpermissive temperature with this mutant produce normal amounts of viral RNA and protein. In addition, sedimentable intracellular viral envelope proteins and nucleoprotein of *ts* 45 were made at 39 C, as they were at permissive temperature; also, viral antigen appeared at the surface of cells infected at 39 C and budding virions could be easily demonstrated by electron microscopy. By comparison, the defect in RNA$^+$ mutant *ts* 23 at nonpermissive temperature was clearly in synthesis of envelope protein S. Similar observations of diminished synthesis of protein S at 40 C have been made by Florence Lafay (*personal communication*). Unlike *ts* 45, cells infected with *ts* 23 at 39 C produced no virions detectable by electron microscopy, but, as was true for *ts* 45, all of the newly synthesized structural proteins of *ts* 23 were largely found to be in a

sedimentable form and viral antigen was readily detected on the surface of plasma membrane. It would appear from these studies that deficiency of protein S in cells infected at 39 C with *ts* 23 results in maturation arrest at a stage in virion assembly beyond insertion of protein G into cell membrane. Consistent with this hypothesis is the data of Lafay and Berkaloff (13) on the thermolability of *ts* 23, which may influence restriction of virion development observed at 40 C.

Mutant *ts* 52 turned out to be the most complex and perhaps the most interesting of the RNA^+ mutants of VS virus. The primary deficiency appears to be failure of glycosylation of the G protein at nonpermissive temperature. The original basis for this postulate was the finding that cells infected at 39 C with *ts* 52 synthesized a protein (G'), the molecular weight of which was about 4,000 to 5,000 daltons less than that of the glycosylated G protein made under permissive conditions. Burge and Huang (2) and McSharry and Wagner (15) have presented evidence that the covalently bonded glycopeptide of VS viral protein G has a molecular weight of about 4,000 daltons; the failure of this polysaccharide chain to attach at nonpermissive temperature could result in accumulation of a nonglycosylated protein similar to that of the G' precursor protein of *ts* 52. Differential incorporation of ^3H-glucosamine at 31 and 39 C served to demonstrate defective glycosylation of G' protein at the nonpermissive temperature. Restrictive glycosylation at the cellular level appears to be ruled out by the finding that sugar transferases function to charge glycolipids made in infected cells at the nonpermissive temperature. A block in the sugar acceptor function of mutant viral protein G' seems to be a more likely explanation for the defect.

This defective glycosylation of protein G' apparently results in a series of secondary effects in cells infected with *ts* 52 at nonpermissive temperature. Not only did protein G' remain in a nonsedimentable form, indicative of failure to be inserted into cell membrane (26), but the nucleoprotein also remained soluble, suggesting deficiency of aggregation into nucleocapsid. Additional evidence of a block in insertion of protein G (and perhaps protein S) into plasma membrane was failure of VS viral antigen to appear on the surface of cells infected with *ts* 52 at 39 C. The RNA^+ mutants *ts* 45 and *ts* 23, representing two other complementation groups, both induced the formation of viral surface antigen at nonpermissive temperature. These data suggest, contrary to a previous suggestion (24), that the envelope glycoprotein is the major antigen of VS virus and is primarily responsible for assembly of the viral components.

The genetics and physiology of the enveloped group A arboviruses (3, 22) appear to be closely analogous to those of VS virus. The *ts* mutants of Sindbis virus have been classified as RNA^- and RNA^+; two complementation groups have been identified among RNA^- mutants, and RNA^+ mutants have been subdivided into three complementation groups (3). RNA^+ arbovirus mutants grown at nonpermissive temperature undergo maturation arrest (21, 29). Deficient or defective proteins have been identified at nonpermissive temperatures for two of the three complementation groups of RNA^+ mutants of Sindbis virus; mutants of complementation group C are deficient in synthesis of Sindbis nucleoprotein (20), whereas those of group D synthesize a defective envelope protein as judged by failure of goose erythrocytes to hemadsorb to infected cells (4) and by thermolability of viral hemagglutinin (28). However, the defective envelope protein of group D Sindbis virus *ts* mutants has electrophoretic mobility identical to that of wild-type envelope protein (20). On the other hand, Lomniczi and Burke (14) reported the rather startling observation that six RNA^+ *ts* mutants of the closely related Semliki Forest arbovirus failed to synthesize any envelope protein at nonpermissive temperature. Scheele and Pfefferkorn (20) reported unsuccessful attempts to demonstrate a precursor protein of the poliovirus type (10) in cells infected with wild-type Sindbis virus; nevertheless, they proposed, with considerable justification, that disturbance in the normal process of cleavage of a polypeptide precursor as a likely hypothesis for failure to detect the Sindbis nucleoprotein in cells infected with class C mutants at nonpermissive temperature. This explanation does not seem likely for the RNA^+ mutants of VS virus for three reasons. First, although VS mutant *ts* 23 was deficient in synthesis of protein S, no larger (or smaller) peptides were found in cells infected at the nonpermissive temperature. Second, Suzanne Emerson in our laboratory (*unpublished observations*) has failed to detect uncleaved precursors in cells infected with wild-type VS virus under exacting conditions of amino acid analogue substitution. Third, the messenger RNA species of VS virus appear to be short minus strands associated with polysomes rather than intact virion plus strands (8).

These hypotheses concerning synthesis and assembly of the proteins of VS virus are being tested in this laboratory by fractionating cells infected with *ts* mutants into membrane and

polyribosome components to analyze the cellular distribution of viral RNA and protein species.

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