Proteins of Vesicular Stomatitis Virus: Kinetics and Cellular Sites of Synthesis

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Five viral peptides synthesized in L cells infected with vesicular stomatitis (VS) virus were identified by polyacrylamide gel electrophoresis and designated as follows: nucleoprotein N, a membrane glycoprotein G, a membrane surface protein S, and two nonstructural proteins NS1 and NS2. A slowly migrating minor structural protein L also present in infected cells is probably an aggregate. Incorporation of ³H-amino acids into each viral protein could be detected by the 2nd hr after infection and even earlier for protein N which is synthesized in the greatest amount. There was no evidence of regulation of viral protein synthesis at the transcriptive level; nonstructural and structural proteins were synthesized throughout the cycle of infection. Short pulses of ³H-amino acids revealed no uncleaved precursor peptides that could be chased into structural peptides. Proteins N and S were chased into released virions but protein G was apparently incorporated into virions as it was being synthesized. VS viral proteins of infected cells were released by mechanically disrupting cytoplasmic membrane by nitrogen decompression and fractionated by high-speed centrifugation. Protein NS1 was present in the nonsedimentable cytoplasmic fraction throughout the cycle of infection. The nucleoprotein N was recovered primarily from the nonsedimentable fraction early in infection but aggregated into a sedimentable component, presumably the nucleocapsid, later in infection. Proteins G and S were always present in the sedimentable fraction of mechanically disrupted infected cells, presumably in association with plasma membrane. Exposure of infected cells to the membrane-dissolving agent, digitonin, resulted in solubilization of most of protein G and all of protein S but not of protein N. These experiments are compatible with the hypothesis that VS viral proteins G and S are synthesized at and inserted into plasma membrane which envelopes a nucleocapsid core to form the VS virion.

Three major structural proteins of vesicular stomatitis (VS) virus have been identified by disc electrophoresis in neutral polyacrylamide gels (10, 23, 24). A fourth minor virion component (designated protein L, for large) is probably an aggregate of the major proteins (J. J. Holland, *personal communication*) which does not dissociate in 8 M urea (23). Controlled degradation of purified VS virions with deoxycholate or digitonin provided evidence for the location in the VS virions of the major proteins and their association with the structural components recognizable by electron microscopy [24; R. R. Wagner and C. A. Schnaitman, *In R. M. Barry and B. W. J. Mahy (ed.), The Biology of Large RNA Viruses*, Academic Press Inc., *in press*]. An internal stacked helical nucleocapsid core is

composed of a single strand of ribonucleic acid (RNA) and a single peptide of molecular weight $\cong 59,500$, hereby designated protein N. The nucleocapsid is surrounded by a trilayered membrane (envelope) composed of lipid and two leaflets of protein from which protrude spikes. This complex membrane structure (including spikes) contains two peptides, one of molecular weight $\cong 81,500$ designated protein G (for glycoprotein, J. Mudd and D. F. Summers, *personal communication*); the other membrane protein of molecular weight $\cong 34,500$ is solubilized by digitonin and is designated protein S (for surface). We are not yet certain which of the two membrane proteins comprises the spikes.

The present report describes the cellular synthesis of the three structural proteins as well as two newly identified nonstructural proteins, all of which appear to be encoded by the VS virus

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genome. These experiments were greatly assisted by the fortuitous finding that infection of cells with unpurified VS virus at relatively high multiplicity rapidly switches off the synthesis of cellular proteins (S. Yamazaki and R. R. Wagner, *in preparation*).

MATERIALS AND METHODS

The materials and many of the procedures were similar to those described in preceding papers of this series (23, 24) and are summarized below.

Virus, cells, and media. The Indiana serotype of VS virus was used throughout. Infectivity titers per milliliter of stock inocula grown in chick embryo fibroblasts or L cells did not vary significantly from 10^8 to 2×10^9 plaque-forming units (PFU). Monolayer cultures of L cells were grown to confluency in plastic petri plates (100 mm in diameter) or plastic flasks (75 cm² surface) with medium 199 and 10% calf serum. Media used for experimental infection were basal medium, Eagle's (BME) with or without leucine and tyrosine or Eagle's minimal essential medium (MEM), both purchased from Grand Island Biological Co., Grand Island, N.Y.

Chemicals. ³H-leucine (10.3 to 40.0 c/mmole), ³H-tyrosine (28.2 to 43 c/mmole), reconstituted uniformly labeled ¹⁴C-amino acids (54 mc/matom), ¹⁴C-arginine (312 mc/mmole), and D-[1-¹⁴C]-glucosamine (2.94 mc/mmole) all were obtained from Schwarz BioResearch Inc., Orangeburg, N.Y.; crystalline digitonin was obtained from Calbiochem, Los Angeles, Calif.

Marker ¹⁴C-proteins of VS virions. As previously described (23, 24), the structural proteins of VS virions were labeled by growing them in L cells for 8 hr in the presence of ¹⁴C-amino acids, 1.13 μ c per ml of BME diluted 1:50 in 5 ml of Earle's saline solution without serum. A 5-ml amount of complete MEM was added at 8 hr and the media were harvested at 17 hr. Virus was concentrated and purified by differential and rate zonal centrifugation (23). Proteins were extracted by sequential treatment with glacial acetic acid, 0.5 M urea, and 1% sodium dodecyl sulfate (SDS). After overnight dialysis against 400 ml of phosphate buffer (pH 7.2), containing 0.5 M urea, 0.1% SDS, and 0.1% 2-mercaptoethanol, the samples were applied to polyacrylamide gels or stored at -60 C for future experiments. The extracted protein markers were quite stable when frozen.

Infection, labeling, and protein extraction of L cells. Monolayer cultures of L cells were first exposed for 1 hr to 10 ml of medium 199 containing actinomycin D (0.5 μ g/ml). Drained cultures were then infected with 0.5 ml of VS virus at a multiplicity of 50 to 100. The cell layers were then washed and covered with 5 ml of special BME devoid of unlabeled leucine and tyrosine and free from serum. A mixture of ³H-leucine and ³H-tyrosine was incorporated in the labeling medium or added at intervals after infection to provide a concentration of 10 or 20 μ c/ml of each amino acid. Cell monolayers to be harvested were drained free from medium, washed twice with large volumes of phosphate-buffered saline (PBS), and re-

drained. The cells in each monolayer were then scraped into 0.4 ml of Earle's saline solution with a rubber or plastic policeman and the suspension was stored at -60 C. Dounce homogenization of cells provided no particular advantage in these experiments and was abandoned in favor of extraction of proteins from whole cells. Proteins were extracted from whole cells or cell fractions by the method of Maizel (11) in precisely the same way as they were from purified VS virions (23). After adding one-tenth volume of glacial acetic acid, the suspension was made 0.5 M with respect to urea and 1% with respect to SDS. After incubation for 1 hr at 37 C, the disrupted cell suspension was dialyzed at room temperature for 16 to 18 hr against 400 ml of phosphate buffer (pH 7.2, 0.01 M) containing 0.1% SDS, 0.5 M urea, and 0.1% 2-mercaptoethanol. Samples of 20 or 50 μ liters were assayed for total radioactivity by scintillation spectrometry.

Polyacrylamide gel electrophoresis. The procedures were essentially the same as those used previously for analyzing virion structural proteins (23), with the exception that visible bands in gels stained with Coomassie blue were not scanned spectrophotometrically because of the large amount of inert cellular protein in the samples applied to the gels. Therefore, complete reliance for identifying viral proteins was placed on migration of ³H-proteins coelectrophoresed with ¹⁴C-protein markers extracted from purified virions. Gels were all composed of 7.5% acrylamide and 0.2% *N,N'*-bis-methylene acrylamide in 0.1 M phosphate at pH 7.2; the gels and both reservoirs also contained 0.5 M urea and 0.1% SDS. Cellular protein samples of 100 μ liters each, containing as much as 1.3 mg of protein (determined by the method of Lowry), were layered on gels 10 cm in length and electrophoresed for 5 to 6 hr at 5 ma/gel constant current, at which time tracking dye had reached the bottom of the gel. This amount of protein in the samples did not appear to overload the gels. Gels were sliced transversely into lengths of 1.25 mm and each slice was dissolved in 0.5 ml of *N*-chlorosuccinimide at 60 C for 2 hr and diluted in toluene-based fluors; the disintegrations were counted by scintillation spectrometry. In double-labeling experiments, ³H and ¹⁴C disintegrations were calculated by the channels ratio method assisted by a computer program.

RESULTS

Incorporation of different amino acids. Preliminary experiments were performed to determine whether certain amino acids are preferentially incorporated into any of the major structural proteins of VS virus. Previous studies (23, 24) did not reveal differential uptake of ³H-leucine plus ³H-tyrosine compared with a mixture of uniformly labeled ¹⁴C-amino acids. Arginine and lysine were chosen for comparison with the leucine-tyrosine mixture of high specific activity because of the possibility that virion protein N (the nucleoprotein) might be a basic protein.

Figure 1 shows that the proportion of ^{14}C -arginine or ^{14}C -lysine incorporated into VS virion proteins L, G, N, and S did not differ from that of ^3H -leucine plus ^3H -tyrosine. Therefore, the nucleoprotein does not appear to be more basic than the other proteins. The data also suggest that incorporation of the leucine-tyrosine mixture of high ^3H specific radioactivity should provide a reliable indication of rates of synthesis of the individual viral proteins.

Selective incorporation of glucosamine. The hypothesis that the G protein is located in the virion membrane (24) was strengthened by evidence that it is a glycoprotein. Figure 2 demonstrates that purified VS virions grown in the

FIG. 1. Comparative incorporation into VS virion peptides of ^{14}C -lysine (A) or ^{14}C -arginine (B). Monolayer cultures of L cells were infected with VS virus at a multiplicity of ~ 100 and incubated for 8 hr in 5 ml of special BME without leucine, tyrosine, or serum but containing ^3H -leucine ($10 \mu\text{C}/\text{ml}$), ^3H -tyrosine ($10 \mu\text{C}/\text{ml}$), and either ^{14}C -lysine ($0.5 \mu\text{C}/\text{ml}$) or ^{14}C -arginine ($0.5 \mu\text{C}/\text{ml}$). A 5-ml amount of complete MEM with 2% calf serum was then added to each culture, and the media were harvested at 17 hr after infection. Labeled released virions were concentrated and purified by differential and rate zonal centrifugation in 0 to 40% sucrose gradients, as previously described (23). Proteins were extracted from virions and analyzed by electrophoresis on urea-SDS acrylamide gels. The results are expressed as the percentage of ^{14}C disintegrations and corrected ^3H disintegrations in each gel segment. Total counts/min recovered from each gel were: (A) ^{14}C , 1,590 and ^3H , 27,155; (B) ^{14}C , 2,330 and ^3H , 28,060.

FIG. 2. Differential incorporation of ^{14}C -glucosamine into structural proteins of VS virions. L-cell monolayers infected with VS virus were incubated in 5 ml of BME diluted 1:50 in Earle's saline solution containing $1 \mu\text{C}$ of D-[1- ^{14}C]-glucosamine per ml. After 8 hr, 5 ml of complete BME was added to each culture; the media were harvested 17 hr after infection. Virions were concentrated and purified by sequential differential, rate zonal (sucrose), and equilibrium (CsCl) centrifugation. Proteins extracted from virions were coelectrophoresed on the same SDS-acrylamide gel along with marker proteins of purified VS virions labeled with ^3H -amino acids.

presence of ^{14}C -glucosamine were preferentially labeled in protein G. The slight amount of radioactivity above background in the region of protein N may, however, not be due to spillover of ^3H into the ^{14}C channel in this double-labeling experiment. Repeat electrophoresis of ^{14}C -glucosamine-labeled virion proteins without marker ^3H -proteins revealed a similar low level of counts in the expected region of the N protein. Nevertheless, protein G is clearly a glycoprotein.

Synthesis and cellular accumulation of VS viral proteins. The kinetics of synthesis of VS viral proteins was studied by infecting L cells at high multiplicity ($m \cong 50$) and incubating the monolayer cultures in the presence of ^3H -leucine and ^3H -tyrosine, each at a concentration of $10 \mu\text{C}/\text{ml}$. Proteins were extracted from suspensions of whole cells harvested at hourly intervals after virus adsorption and analyzed by disc electrophoresis along with ^{14}C -protein markers extracted from purified VS virions.

Figure 3 shows the electropherograms of the ^3H -proteins recovered from 3.6×10^7 L cells (two monolayer cultures) at hourly intervals after infection. By 1 hr, small peaks of ^3H radioactivity could be identified in the region of G and N ^{14}C -protein markers. The S protein was detected in cells harvested at 2 hr. By the 3rd hr after infection, all three major structural proteins were clearly identifiable and there was little increase in the height of the peaks thereafter. Protein L, the aggregate or large virion peptide

FIG. 3. Electropherograms of VS viral ^3H -peptides extracted from L cells harvested at hourly intervals after infection at a multiplicity of ~ 50 . A 5-ml amount of special BME with $10 \mu\text{C}/\text{ml}$ each of ^3H -leucine and ^3H -tyrosine was added to washed cultures after 30 min of viral adsorption. At each hour after infection, the cells from two cultures ($\sim 4 \times 10^6$ cells) were washed with 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 ^3H -proteins at each time period 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. Also shown are the positions of two nonstructural viral proteins, NS1 and NS2. None of these peptide peaks could be found in uninfected control L cells labeled with ^3H -amino acids for 1 or 5 hr.

(23, 24), was also present at each interval after infection.

Two nonstructural proteins designated NS1 and NS2 could be identified in VS virus-infected cells (Fig. 3). These proteins are presumed to be of viral origin because similar ^3H -protein peaks could not be found in labeled uninfected cells (Fig. 5). NS1 was produced in relatively large amounts, whereas NS2 was present in small amounts and sometimes could not be detected, particularly when the background radioactivity was too high. The molecular weights of these two nonstructural proteins were estimated by the method of Shapiro et al. (17), as previously described for the structural proteins of VS virus (23). NS1 migrated as a peptide of molecular weight equivalent to $\sim 55,000$ daltons and NS2 migrated as a peptide of molecular weight equivalent to $\sim 30,000$ daltons.

Figure 4 compares the rates of synthesis and accumulation in infected L cells of the individual viral peptides. These data were obtained by measuring the area under each ^3H -peak shown at each time interval in Fig. 3 with a compensating

polar planimeter (Gelman Instrument Co., Ann Arbor, Mich.). The total area measured under each electropherogram provided an estimate of total protein synthesized at each time. Studies to be published separately (S. Yamazaki and R. R. Wagner, *in preparation*) revealed that at least 80% of the total cellular ^3H -protein was of viral origin; infection with VS virus at this multiplicity rapidly switches off cellular protein synthesis. Figure 4 demonstrates that the rate of synthesis of all viral peptides reached a plateau at 3 hr after infection. The increase in proteins G and N between 5 and 6 hr was not always apparent (Fig. 5). Also, clearly evident in Fig. 4 are the markedly different amounts of each viral protein synthesized despite the similarity in their rates of synthesis.

Pulse labeling of viral proteins. One-step growth curves of VS virus reveal a latent period of 2 hr followed by exponential production and release of infectious virus to a peak titer at 6 to 8 hr (22). More definitive data on the onset and duration of synthesis of each viral protein during the viral growth cycle could be obtained by pulse

FIG. 4. Relative concentrations of individual and total VS viral ^3H -proteins in L cells at intervals after infection. The data represent the area under each protein peak shown for the six time periods in Fig. 3, as measured with a compensating planimeter. Total ^3H -proteins represent the area under the entire curve for each hourly period.

labeling. To this end, 15 monolayer cultures of L cells were infected with VS virus at a multiplicity of 50 and labeled by 1-hr pulses of ^3H -leucine plus ^3H -tyrosine at 1-hr intervals after virus adsorption. The cells from three monolayers ($\cong 5 \times 10^7$ cells) were harvested after each pulse and the proteins were extracted from unfractionated cells for analysis by electrophoresis on SDS-urea acrylamide gels along with ^{14}C -protein markers extracted from purified virions. Uninfected cells pulsed for 1 hr with ^3H -leucine plus ^3H -tyrosine served as controls.

Figure 5 demonstrates failure to detect viral proteins within the 1st hr after infection with the possible exception of protein N. However, the background radioactivity of nonviral cellular proteins in this experiment was probably too high at 1 hr to detect small amounts of viral protein. The uninfected cell control shown in Fig. 5 illustrates the very high level of ^3H -amino acid incorporation into cellular protein in 1 hr compared with the marked reduction of total protein synthesis in infected cells. Beginning synthesis of all viral structural proteins and NS1 could be demonstrated 1 to 2 hr after infection. Although incorporation of ^3H -amino acids into

FIG. 5. Comparative rates of incorporation of ^3H -amino acids pulsed into L cells at 1 hr intervals after infection with VS virus at a multiplicity of ~ 50 . ^3H -leucine and ^3H -tyrosine ($10 \mu\text{C}/\text{ml}$ each) were added to culture medium for 1 hr at the times shown. The cells were harvested from three cultures ($\sim 5 \times 10^6$ cells) at the end of each pulse, and the proteins were extracted from whole cells and analyzed by disc electrophoresis along with marker ^{14}C -proteins of purified VS virions, as described for the experiment shown in Fig. 3. Note the very large amount of ^3H -protein and amorphous electropherogram of the control uninfected cells also pulsed for 1 hr with the same amount of ^3H -amino acids. Arrows indicate migration of virion marker ^{14}C -protein peaks; NS1 can usually be seen as a leading shoulder on peak N but NS2 is difficult to identify.

all viral proteins appeared to be maximal between 3 and 4 hr, synthesis of the three structural proteins and the two nonstructural proteins continued for at least 5 to 6 hr. The apparent decline in the rate of viral protein synthesis 3 hr after infection shown in Fig. 3 and 4 could be due to depletion of ^3H -amino acid precursors under conditions of prolonged incubation.

These data suggest absence of regulation in the order of VS viral cistrons which code for each individual peptide. The earlier detection of structural protein N may well be due to its greater rate of synthesis throughout the infectious cycle. Protein NS1, which is presumably a viral enzyme, did not appear before the structural proteins and its synthesis did not switch off late in infection.

Pulse-chase experiments. These experiments were designed to answer two questions. (i) Is there any evidence for a large precursor peptide read off intact from the entire polycistronic message of VS virus which is later cleaved into individual structural peptides as in the case of poliovirus protein synthesis (5, 9, 20)? (ii) Although the structural proteins of VS virus appear to be synthesized at about the same rate and throughout the cycle of infection, are each of the three major structural peptides incorporated into virions and released from the cell at the same rate?

To explore further the first question (the possibility of a precursor peptide), experiments were performed in essentially the same manner as those of Jacobson and Baltimore (9) with poliovirus-infected cells. L cells were infected with VS virus at a multiplicity of ~ 100 and incubated in 5 ml of BME without leucine and tyrosine. At 3, 3.5, or 5 hr after infection, each culture was pulse-labeled for 3 min with $20\text{ }\mu\text{C}/\text{ml}$ each of ^3H -leucine and ^3H -tyrosine. To stop the pulse, cultures were washed with BME containing added 5 mg/ml each of unlabeled leucine and tyrosine; others were chased by reincubation for 30, 60, 90, or 120 min with the unlabeled leucine and tyrosine chase medium. The cells from two cultures ($\sim 3.5 \times 10^7$ cells) were harvested and the ^3H -peptides were extracted and analyzed by disc electrophoresis in SDS-urea gels along with ^{14}C -marker virion proteins.

The results were completely negative. There was no evidence for an uncleaved precursor in the infected cells pulsed for 3 min. Approximately the same number of ^3H counts were present in peptides L, G, N, NS1, S, and NS2 after pulsing for 3 min and after each chase period.

Similar experiments were performed to estimate the rate at which the structural peptides are incorporated into released virions. The cells were infected in the same manner for 3.5 or 5 hr, pulsed

for 30 min with $20\text{ }\mu\text{C}/\text{ml}$ of ^3H -amino acids, and chased for 0, 1, 2, or 3 hr with BME containing added unlabeled leucine and tyrosine. The cells from two plate cultures were examined for VS viral proteins. Medium from five cultures was also harvested and pooled after each chase period; the released virions were concentrated by centrifugation and the structural proteins were extracted and examined by acrylamide gel electrophoresis. The electropherograms of released virions at each chase interval were compared by measuring the area under each peak with the Gelman compensating planimeter.

Figure 6 shows the comparative amounts of each of the ^3H -proteins present in virions released from infected L cells after progressive incubation

FIG. 6. Cellular VS viral ^3H -peptides chased into VS virions. Twenty cultures of L cells ($\sim 1.8 \times 10^7$ cells/culture) were infected with VS virus at a multiplicity of ~ 100 and incubated in BME free from leucine and tyrosine for 3.5 hr. All cultures were then pulsed with ^3H -leucine and ^3H -tyrosine ($20\text{ }\mu\text{C}/\text{ml}$ each) for 30 min. The medium was removed from five cultures at the end of the 30-min pulse (0 chase). The remaining 15 cultures were washed and reincubated in complete BME with added unlabeled leucine and tyrosine (2 mg/ml each), and the media from five cultures were harvested at hourly intervals after the pulse. The virions from the pulse-labeled cultures and those chased for 1, 2, and 3 hr were concentrated by centrifugation at $100,000 \times g$. The proteins were extracted as in other experiments and analyzed by disc electrophoresis. The area under each ^3H -protein peak in the electropherogram was determined with the aid of a compensating planimeter. The scale is equivalent to only one-tenth of that shown in Fig. 4.

in unlabeled chase medium. At the end of the 30-min pulse period (0 chase), protein G was released in greatest amount but did not increase significantly during the successive periods of chase. In contrast, protein N was barely detectable in released virions at time 0 but progressively increased with each chase, as did protein S but to a somewhat lesser extent. The efficiency of the chase procedure was demonstrated by finding no increase in any of the intracellular viral proteins from the same cultures at corresponding periods of chase with cold amino acids.

These results suggest that virion protein G is incorporated rapidly after synthesis, whereas protein N forms an intracellular pool which is only gradually incorporated into virions budding from the cell surface. Protein S, the other viral membrane protein, appears to be intermediate between G and N. One possibility is that incorporation of protein G is the final step in assembly of the virion which is then released from the cell surface.

Fractionation of viral proteins after mechanical disruption of infected cells. These experiments were based on the observation that VS virions are associated with and bud from plasma membrane (14) or other membranous organelles (3) of the host cell. It seemed likely that synthesis or assembly of structural proteins might occur at the plasma membrane. It was of interest, therefore, to fractionate infected cells to investigate the hypothetical association of viral proteins with cellular membrane.

L-cell monolayers pretreated with actinomycin were infected as before with VS virus at a multiplicity of 50 and then labeled with ^3H -leucine and ^3H -tyrosine for 2, 3, or 4 hr. The cells were then washed with PBS and scraped into 2 ml of "mitochondrial" medium (16) containing 210 mM D-mannitol, 70 mM sucrose, 2 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid) buffer, 0.05% bovine serum albumin, and 1 mM MgSO_4 . The nitrogen bomb method described by Wallach and Kamat (25) was adapted for stripping off the plasma membrane of L cells. Wu et al. (27) successfully adopted this technique for separating plasma membranes of transformed and untransformed cells to compare their glycoproteins.

Suspensions of L cells were equilibrated for 20 min at 800 psi in a nitrogen pressure homogenizer (Parr Instrument Co., Moline, Ill.) followed by rapid return to atmospheric pressure. Approximately 1.3 ml of each disrupted cell suspension was recovered after expulsion from the nitrogen bomb. J. W. Heine and C. A. Schnaitman of this department have found that this procedure releases maximum amounts of glucose-6-phos-

phate dehydrogenase and minimal amounts of lysosomal acid phosphatase. The cell suspensions were then made 10^{-2} M with respect to disodium ethylenediaminetetraacetate (EDTA), and the nuclei and other gross cellular debris were removed by centrifugation at $1,000 \times g$ for 15 min. The cytoplasmic contents were then centrifuged at $130,000 \times g$ for 90 min in special 1-ml tubes designed to fit the SW 65 rotor. The supernatant fluids, representing soluble cytoplasmic constituents, were drawn off and the pellets, which contained membranes as well as other particulate material, were resuspended with the aid of sonic oscillation in 0.5 ml of Earle's saline solution. Proteins were extracted from the supernatant fluid and resuspended pellet fractions with acetic acid, 0.5 M urea, 1% SDS, and 2-mercaptoethanol for analysis by electrophoresis in SDS-urea polyacrylamide gels along with virion ^{14}C -protein markers.

Figure 7 shows the proportions of nonsedimentable and sedimentable VS viral proteins recovered from mechanically fractionated L cells at 2, 3, and 4 hr after infection. The nonstructural protein NS1 was found exclusively in the nonsedimentable cell fraction throughout the cycle of infection, except for trace amounts in the sediment which probably represent contamination of the pellet. Structural protein N was essentially nonsedimentable up to 2 hr after infection, but approximately half of the intracellular protein N was recovered in the sedimentable fraction thereafter. In sharp contrast, proteins G and S could not be detected in the nonsedimentable cell fraction at any stage after infection but were present in the sedimentable fraction in increasing amounts as the infection progressed. Protein L was present in somewhat higher concentration in the sedimentable than in the nonsedimentable fraction. Protein NS2 was recovered in insufficient amounts to be significant but appeared to be soluble.

These cell-fractionation experiments can be interpreted provisionally as follows. (i) Protein NS1, as expected of a nonstructural protein, is synthesized in the cytoplasm, does not form large aggregates, and remains unassociated with cell membrane or other cellular particulate material. (ii) Protein N is synthesized in the cytoplasm to form a large intracellular pool of monomers or oligomers which subsequently aggregate (presumably with progeny RNA) to form a sedimentable nucleocapsid. These experiments provide no evidence whether the nucleocapsid becomes membrane-bound. (iii) Proteins G and S are either synthesized on free polysomes in the cytoplasm whence the newly synthesized proteins are rapidly bound to membrane or, more likely,

FIG. 7. Relative amounts of VS viral ^3H -proteins associated with nonsedimentable (●) and sedimentable (○) fractions of L cells disrupted with the nitrogen bomb at 2, 3, and 4 hr after infection with VS virus. Monolayer cultures of $\sim 2 \times 10^7$ cells each were infected with VS virus at a multiplicity of ~ 50 and incubated in special BME with $10 \mu\text{C}/\text{ml}$ each of ^3H -leucine and ^3H -tyrosine. At 2, 3, and 4 hr after infection, cells from a single culture were scraped into 2 ml of "mitochondrial medium" and disrupted by sudden release of 800 psi of equilibrated nitrogen pressure. The nuclei and gross cellular debris were removed by centrifugation at $1,000 \times g$ for 15 min. The cytoplasmic contents were then separated into nonsedimentable and sedimentable fractions by centrifugation at $130,000 \times g$ for 90 min. The proteins were extracted from the supernatant fluid and pellet and analyzed on acrylamide gels along with virion marker ^{14}C -proteins (L, G, N, and S).

they are synthesized on membrane-associated polysomes and are inserted directly into the plasma membrane. The following experiment supports the evidence that G and S proteins are membrane-associated.

Solubilization of cellular G and S proteins with digitonin. If the G and S proteins are inserted into the plasma membrane of L cells infected with VS virus, they should be solubilized by digitonin. Digitonin is a sterol glycoside that complexes with cholesterol and has been used to strip off the outer membrane of mitochondria (16) and to solubilize plasma membrane. Earlier experiments had shown that digitonin liberates in a soluble form about 80% of protein S and 50% of protein G from purified VS virions (24).

L cells infected with VS virus at a multiplicity of 50 were incubated for 5 hr in BME containing ^3H -leucine and ^3H -tyrosine. The monolayers of 1.8×10^7 cells each were washed with buffered saline, drained, and scraped into 0.4 ml of ice-cold Earle's saline solution. Crystalline digitonin dissolved in 0.1 ml of distilled water was added to each suspension of scraped cells at concentrations of 0.2, 0.4, or 0.6 mg/mg of protein. After incubation with digitonin for 10 min at 4 C, the disrupted cells were centrifuged at $130,000 \times g$ for 90 min at 4 C. The supernatant fluid was then drawn off and the drained pellet was resuspended in 0.5 ml of Earle's saline solution. Proteins were extracted from the supernatant fluids and resuspended pellets and analyzed by electrophoresis on 7.5% acrylamide gels containing SDS and urea.

Figure 8 compares the electropherograms of sedimentable and soluble viral ^3H -proteins present in infected L cells disrupted with 0.4 mg of digitonin 5 hr after infection. Similar results were obtained with 0.2 or 0.6 mg of digitonin. All of protein S and approximately 75% of protein G were recovered in the nonsedimentable fraction of digitonin-disrupted cells. This result compares with complete recovery of proteins G and S in the sedimentable fraction of mechanically disrupted cells (Fig. 7). By comparison, much of protein N was pelleted after digitonin treatment of 5-hr infected cells, a finding which supports the hypothesis that much of protein N is assembled into sedimentable nucleocapsids by 5 hr. Similar results were obtained when purified virions were treated with digitonin (24). As expected, both nonstructural viral proteins were found in the soluble fraction of digitonin-disrupted cells.

This experiment was repeated by exposing cultures of 2×10^7 infected L cells to 0.4 mg of digitonin at 2, 3, or 4 hr after infection. The results were similar to those shown in Fig. 8. The pelleted fractions contained only traces of protein G and no protein S, both of which were

FIG. 8. Distribution of VS viral ^3H -proteins in nonsedimentable ($\bullet$), and sedimentable ($\circ$) fractions of L cells treated with digitonin 5 hr after VS viral infection. The infected cells labeled with ^3H -amino acids were scraped into 0.4 ml of cold Earle's saline solution and treated with digitonin (0.4 mg digitonin/mg of protein). The disrupted cells were then centrifuged at $130,000 \times g$ for 90 min. Proteins extracted from the supernatant fluid and pellet were analyzed on separate acrylamide gels along with marker ^{14}C -proteins extracted from purified virions (arrows).

recovered in the digitonin-solubilized cell fraction at each interval after infection.

These data suggest that proteins G and S are integral components of the plasma membrane of VS virus-infected L cells and are liberated by digitonin presumably through its interaction with membrane cholesterol.

DISCUSSION

Certain similarities and differences in protein composition and synthesis among the RNA viruses are becoming apparent. Newcastle disease virus is strikingly similar to VS virus in that the virions contain two membrane proteins and one core protein, each of which has a molecular weight very similar to that of the corresponding three structural proteins of VS virus (4). Similar polypeptides are present in influenza (21) and parainfluenza SV5 (1) virions. Arboviruses of group A, which are also membrane-enclosed but structurally quite different from VS virus, myxoviruses, and paramyxoviruses are composed of two proteins (19). The membrane of the Sindbis arbovirus apparently contains only a single protein, which, however, splits into two peaks unless disulfide bonds are completely reduced (18). Similar split peaks are sometimes seen in the region of the VS viral G protein (10, 23).

An advantage of VS virus for studying viral protein synthesis is the rapidity with which cellular protein synthesis is switched off after infection.

One possible disadvantage of our system is the necessity for using high multiplicities of infection; at multiplicities lower than 10, inhibition of cellular protein synthesis is insufficient to detect early viral protein synthesis (Wagner, *unpublished data*). This inhibition of cellular protein synthesis could not be reversed by interferon at a concentration which completely blocks synthesis of viral proteins (S. Yamazaki and R. R. Wagner, *in preparation*). Nor does synthesis of new viral protein appear to be essential for the rapid inhibition of cellular RNA synthesis (7). By comparison, infection with Sindbis virus at about the same multiplicity results in maximal inhibition of cellular protein synthesis only at 2 to 3 hr (18).

Only two nonstructural viral proteins could be definitively identified in cells infected with VS virus. This does not include the variable peaks that migrate slowly; these are undoubtedly aggregates of viral or cellular protein. We also exclude from consideration the constant L protein which is present in virions as well as infected cells. The molecular weight of the L protein has been estimated at 275,000 daltons (23), which is equal to the combined molecular weights of all five structural and nonstructural proteins. The L protein of VS virus was not shown to be an uncleaved peptide precursor of the five smaller proteins, as in the case of poliovirus (5, 9, 20), but it is more likely to be an aggregate. Pulse-chase experiments to demonstrate cleavage of L protein into smaller peptides were negative. If the L protein is assumed to be an aggregate and discounted, the combined molecular weight of 260,000 estimated for the five definite VS viral peptides is insufficient to account for all of the information potentially encoded by the VS virus genome of $\sim 3.6 \times 10^6$ daltons (8). By comparison, 13 or 14 separate viral peptides found in cells infected with poliovirus (12) or Sindbis virus (18) in each case far exceed the genetic potential of these viruses. There seems to be less risk in the VS virus system of confusing authentic viral peptides with uncleaved precursors and nonviral proteins.

There are several striking features of the kinetics of synthesis of VS viral proteins. The first is that all of the viral proteins, nonstructural as well as structural, appear in infected cells at about the same time and are synthesized at about the same rate. The predominant protein N and possibly proteins G and NS1 could be detected in the 1st hr, but all five proteins were present during the 2nd hr, and synthesis of each of them was maximal between 2 and 4 hr after infection. The pulse experiments show some decline in synthesis of all viral proteins late in infection. No switch off of

nonstructural proteins could be discerned. Therefore, these studies strongly suggest lack of regulation at the transcriptive level in the synthesis of VS viral peptides. In contrast, vaccinia virus (6) and adenovirus (26) show ordered synthesis of different classes of structural peptides. The core peptides of adenovirus are arginine-rich (13) and are produced early but in smaller amounts than the capsid peptides (26). The core protein (N) of VS virus did not appear to be rich in arginine or lysine and is synthesized in amounts far in excess of that needed for nucleocapsid assembly.

Another striking feature of VS viral protein synthesis is the disparity in the amount of each protein that is made and accumulates in the cell. Protein N, for example, forms a very large pool compared with protein S, which is present in relatively small amounts in cells at any time. By comparison, radioactive protein S is present in released virions in proportions at least half that of the predominant protein N. Considering their differences in molecular weight, the completed virion probably contains equivalent numbers of molecules of proteins N and S. Assuming lack of regulation at the transcriptive level, the differences in amounts of each protein synthesized must be determined at the translational level.

The cellular sites of synthesis and accumulation also appear to differ for the various VS viral proteins. Protein NS1 clearly remains free in the cytoplasm unassociated with cellular organelles and does not aggregate to any significant extent. The nucleoprotein (N) appears first in a soluble form, presumably free in cytoplasm, and then becomes sedimentable. It seems likely that this sedimentability is not merely attributable to association with cell membrane because protein N is not solubilized by digitonin. In the latter stages of infection, almost half of protein N is in a soluble cytoplasmic pool and the rest is presumably aggregated with progeny RNA into growing nucleocapsids that are inserted into virions. Proteins G and S were never found free in the cytoplasm and appear to be always associated with cell membrane, presumably plasma membrane. Neither of the two viral membrane proteins could be recovered in the supernatant fluid of mechanically disrupted cells after high-speed centrifugation. All of protein S and about two-thirds of protein G were solubilized by exposing infected cells to digitonin, in a manner similar to the dissociation of proteins S and G from virions treated with digitonin (24). These data are construed as evidence that both membrane proteins of VS virions are synthesized on and inserted into the cholesterol-containing plasma membrane or another cell membrane, which then becomes the

cholesterol-containing membrane of the VS virion after envelopment of the nucleocapsid.

It is also of interest that no soluble pool of the membrane protein of Sindbis virus can be detected in infected cells [B. W. Burge, quoted by Scheele and Pfefferkorn (15)], presumably indicating association with cellular membrane. Friedman and Grimley (2) have presented evidence that the bulk of protein synthesis of the closely related Semliki Forest virus takes place on membranes, probably rough endoplasmic reticulum, rather than on the plasma membrane. Assembly and release of arbovirions follow rapidly after viral protein synthesis (2, 15).

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LITERATURE CITED

1. Caliguiri, L. A., H.-D. Klenk, and P. W. Choppin. 1969. The proteins of parainfluenza virus SV5. I. Separation of virion polypeptides by polyacrylamide gel electrophoresis. *Virology* 39:460-466.
2. Friedman, R. M., and P. M. Grimley. 1969. Inhibition of arbovirus assembly by cycloheximide. *J. Virol.* 4:292-299.
3. Hackett, A. J., Y. C. Zee, F. L. Schaffer, and L. Talens. 1968. Electron microscopic study of the morphogenesis of vesicular stomatitis virus. *J. Virol.* 2:1154-1162.
4. Haslam, E. A., I. A. Cheyne, and D. O. White. 1969. The structural proteins of Newcastle disease virus. *Virology* 39:118-129.
5. Holland, J. J., and E. D. Kiehn. 1968. Specific cleavage of viral proteins as steps in the synthesis and maturation of enteroviruses. *Proc. Nat. Acad. Sci. U.S.A.* 60:1015-1022.
6. Holowczak, J. A., and W. K. Joklik. 1967. Studies on the structural proteins of vaccinia virus. II. Kinetics of the synthesis of individual groups of structural proteins. *Virology* 33:726-739.
7. Huang, A. S., and R. R. Wagner. 1965. Inhibition of cellular RNA synthesis by nonreplicating vesicular stomatitis virus. *Proc. Nat. Acad. Sci. U.S.A.* 54:1579-1584.
8. Huang, A. S., and R. R. Wagner. 1966. Comparative sedimentation coefficients of RNA extracted from plaque-forming and defective particles of vesicular stomatitis virus. *J. Mol. Biol.* 22:381-384.
9. Jacobson, M. F., and D. Baltimore. 1968. Polypeptide cleavage in the formation of poliovirus protein. *Proc. Nat. Acad. Sci. U.S.A.* 61:77-84.
10. Kang, C. Y., and L. Prevec. 1969. Proteins of vesicular stomatitis virus. I. Polyacrylamide gel electrophoresis of viral antigens. *J. Virol.* 3:404-413.
11. Maizel, J. V., Jr. 1966. Acrylamide-gel electropherograms by mechanical fractionation: radioactive adenovirus proteins. *Science (Washington)* 151:988-990.
12. Maizel, J. V., Jr., and D. F. Summers. 1968. Evidence for differences in size and composition of the poliovirus-specific polypeptides in infected HeLa cells. *Virology* 36:48-54.
13. Maizel, J. V., Jr., D. O. White, and M. D. Scharff. 1968. The polypeptides of adenovirus. II. Soluble proteins, cores, top components and the structure of the virion. *Virology* 36:126-136.
14. Nakai, T., and A. F. Howatson. 1968. The fine structure of vesicular stomatitis virus. *Virology* 35:268-281.
15. Scheele, C. M., and E. R. Pfefferkorn. 1969. Kinetics of incorporation of structural proteins into Sindbis virus. *J. Virol.* 3:369-375.

16. Schnaitman, C., V. G. Erwin, and J. W. Greenawalt. 1967. The submitochondrial localization of monoamine oxidase. *J. Cell Biol.* 32:719-735.
17. Shapiro, A. L., E. Viñuela, and J. V. Maizel, Jr. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochem. Biophys. Res. Commun.* 28:815-820.
18. Strauss, J. H., Jr., B. W. Burge, and J. E. Darnell. 1969. Sindbis virus infection of chick and hamster cells: synthesis of virus-specific proteins. *Virology* 37:367-376.
19. Strauss, J. H., Jr., B. W. Burge, E. R. Pfefferkorn, and J. E. Darnell, Jr. 1968. Identification of the membrane protein and "core" protein of Sindbis virus. *Proc. Nat. Acad. Sci. U.S.A.* 59:533-537.
20. Summers, D. F., and J. V. Maizel, Jr. 1968. Evidence for large precursor proteins in poliovirus synthesis. *Proc. Nat. Acad. Sci. U.S.A.* 59:966-971.
21. Taylor, J. M., A. W. Hampson, and D. O. White. 1969. The polypeptides of influenza virus. I. Cytoplasmic synthesis and nuclear accumulation. *Virology* 39:419-425.
22. Wagner, R. R., A. H. Levy, R. M. Snyder, G. A. Ratcliffe, and D. F. Hyatt. 1963. Biologic properties of two plaque variants of vesicular stomatitis virus. *J. Immunol.* 91:112-122.
23. Wagner, R. R., T. C. Schnaitman, and R. M. Snyder. 1969. Structural proteins of vesicular stomatitis virus. *J. Virol.* 3:395-403.
24. Wagner, R. R., T. C. Schnaitman, R. M. Snyder, and C. A. Schnaitman. 1969. Protein composition of the structural components of vesicular stomatitis virus. *J. Virol.* 3:611-618.
25. Wallach, D. F. H., and V. Kamat. 1964. Isolation of cytoplasmic membranes from Ehrlich ascites tumor cells. *Proc. Nat. Acad. Sci. U.S.A.* 52:721-728.
26. White, D. O., D. M. Scharff, and J. V. Maizel, Jr. 1969. The polypeptides of adenovirus. III. Synthesis in infected cells. *Virology* 38:395-406.
27. Wu, H. C., E. Meezan, P. H. Black, and P. W. Robbins. 1969. Comparative studies on the carbohydrate-containing membrane components of normal and virus-transformed mouse fibroblasts. I. Glucosamine-labeling patterns in 3T3, spontaneously transformed 3T3, and SV40-transformed 3T3 cells. *Biochemistry* 8:2509-2517.