In Vitro Protein-Synthesizing Activity of Vesicular Stomatitis Virus-Infected Cell Extracts

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Crude cytoplasmic extracts from vesicular stomatitis virus (VSV)-infected HeLa cells incorporate radioactive amino acids into hot trichloroacetic acid-precipitable material linearly for 10 to 20 min. The material synthesized *in vitro* corresponds in molecular weight to four of the five VSV structural proteins. However, synthesis of the viral glycoprotein (G) is significantly reduced, whereas the relative amounts of viral structural proteins L and NS synthesized are increased compared with the ratio of the proteins found in the virion. Fractionation of a VSV-infected crude cytoplasmic extract into a cytoplasmic pellet ($20,000 \times g$ for 30 min) and a cytoplasmic supernatant results in a significant reduction in protein synthesizing activity of both fractions, although both contain polysomes. The products synthesized by a cytoplasmic supernatant-directed system included all the VSV structural proteins except the glycoprotein, whereas in an *in vitro* system directed by the cytoplasmic pellet there is a marked reduction in synthesis of the nucleoprotein (N) and also a small relative increase in synthesis of the glycoprotein. Addition of uninfected, preincubated HeLa or L-cell S10 or a HeLa ribosomal fraction to the VSV-infected cytoplasmic pellet results in a 30- to 60-fold stimulation of ^{35}S -methionine incorporation. However, these uninfected extracts do not stimulate ^{35}S -methionine incorporation by the infected crude cytoplasmic extract or the cytoplasmic supernatant. The products synthesized by the stimulated cytoplasmic pellet now include sizeable amounts of the glycoprotein in addition to the other VSV structural proteins.

Vesicular stomatitis virus (VSV) is an RNA-containing membrane-maturing virus which is composed of five structural proteins (11, 14, 23, 26). Two of the structural proteins, G (which is a glycoprotein) (3, 10) and M, are associated with the virion envelope (4, 9, 23). Protein N, the nucleoprotein, is found associated with viral RNA (9, 23). The function of proteins L and NS is unknown, although there is speculation that protein NS may be involved in the functioning of the RNA-dependent RNA polymerase (2, 7, 21) which is an integral part of the virion (1). In addition to the structural proteins, a number of minor viral-specified proteins are present in VSV-infected cells (14, 23).

Kinetics of the appearance of VSV proteins in infected HeLa cells treated with actinomycin D show that viral-specific structural proteins begin to appear before 2 h postinfection and increase in amount up to at least 4 to 5 h (14). Very little, if anything, is known about the

appearance or function of nonstructural viral proteins during the infectious cycle.

We attempted to obtain a better understanding of the mechanisms involved in VSV translation by utilizing an *in vitro* protein-synthesizing system primed by VSV-specific polysomes extracted from infected HeLa cells.

On the basis of a number of criteria we found that this *in vitro* protein-synthesizing system faithfully directed the synthesis of only VSV proteins. However, synthesis of the virion glycoprotein is drastically reduced, whereas synthesis of protein L is increased compared with that observed in infected cells. Fractionation of the crude cytoplasmic extract resulted in a considerable reduction of protein-synthesizing activity by either the cytoplasmic pellet ($20,000 \times g$, for 30 min) or the cytoplasmic supernatant. However, incorporation of amino acids by the cytoplasmic pellet could be stimulated 30- to 60-fold by addition of preincubated S10 extracts

from uninfected HeLa or L cells. All the virion structural proteins, including the glycoprotein (G), are synthesized by the stimulated cytoplasmic pellet. The factor(s) responsible for the stimulation appears to be restricted to the ribosomal fraction of the uninfected extracts.

MATERIALS AND METHODS

Cell and virus cultures. Suspension cultures of HeLa cells were grown in Eagle medium (MEM) (Schwartz Research, Inc.) plus 7% fetal calf serum (Grand Island Biological Co., Inc.) and 2 mM glutamine (Grand Island Biological Co., Inc.) at a concentration of about 4×10^5 to 6×10^5 cells per ml.

VSV was of the Indiana serotype. Both unlabeled and ^3H -amino acid-labeled VSV were grown and purified as previously described (8, 14). After the final purification step, the ^3H -amino acid-labeled virus was precipitated by a series of washes in trichloroacetic acid and finally in acetone. The virus was solubilized in 0.1 M sodium phosphate buffer, pH 7.2, 1% sodium dodecyl sulfate (SDS), and 0.1% 2-mercaptoethanol (solubilizing buffer), and was kept frozen. The virus was then used as a marker on acrylamide gels.

Infection of HeLa cells. Cells, growing at 37 C, were concentrated to 4×10^6 cells per ml in MEM (minus serum) containing 14 mM HEPES buffer (*N*-2-hydroxyl-ethylpiperazine-*N'*-2'-ethane sulfonic acid; Nutritional Biochemical Corp.) and 2 mM glutamine. Actinomycin D (Merck and Co.) was added to a concentration of 5 $\mu\text{g}/\text{ml}$ at 1 h postinfection, and at 1.5 h postinfection fetal calf serum was added to a concentration of 5%. Radioactive uridine (28.5 Ci/mmol, 500 $\mu\text{Ci}/\text{ml}$) (New England Nuclear Corp.) was added at 2 h postinfection and radioactive amino acids (New England Nuclear Corp., Amer-sham/Searle) at 2 to 3 h.

The course of the infection was always traced on a small portion of the infected cell culture by measuring the incorporation of ^{14}C -uridine in the presence of actinomycin D (16). In all experiments (unless otherwise specified) the infection was stopped at 4.5 h postinfection at which time primarily VSV-specific proteins were being synthesized (14).

Preparation of cell extracts. Cells were washed twice in cold Earle solution, resuspended for 10 min in hypotonic buffer, RSB (0.01 M NaCl or KCl, 0.0015 M MgCl_2 , 0.01 M Tris, pH 7.4) containing 50 μg of heparin per ml. The cells were broken in a stainless-steel Dounce homogenizer, and this procedure was traced with a phase-contrast microscope. Unbroken cells and nuclei were pelleted by centrifugation at 1,600 rpm for 3 to 4 min. The supernatant fluid was used as the crude cytoplasmic extract. This extract was separated into a cytoplasmic supernatant and cytoplasmic pellet by centrifugation (Sorvall Angle rotor, SS34) at $20,000 \times g$ for 30 min (16). A ribosomal pellet was prepared from the cytoplasmic supernatant by centrifugation in a Type 65 rotor at 50,000 rpm for 3 h. Both the cytoplasmic pellet and the ribosomal pellet were resuspended in RSB, containing 50 μg of heparin per ml, by homogenization in

a glass Dounce homogenizer. Glycerol was added to each extract to a final concentration of 10%, and extracts were then divided into small samples and frozen at -70°C . The extracts retained protein-synthesizing activity for a number of months.

Extracts from uninfected HeLa and L cells were prepared according to essentially the same procedure as Eggen and Shatkin (6). The cells were collected by low-speed centrifugation and were washed three times in cold buffer A (35 mM Tris-hydrochloride, pH 7.5, and 140 mM NaCl). The packed cells were resuspended in 2 to 3 volumes of 10 mM KCl, 1.5 mM MgCl_2 , 10 mM Tris-hydrochloride, pH 7.5, and 6 mM 2-mercaptoethanol (buffer B) for 10 min at 4 C, and were broken in a Dounce homogenizer. The suspension was centrifuged at $10,000 \times g$ for 10 min and a 0.1-volume of 10-times-concentrated incubation buffer (0.2 M Tris-hydrochloride, pH 7.5, 1.2 M KCl, 0.05 M magnesium acetate and 0.06 M 2-mercaptoethanol) was added. The extract was preincubated at 37 C for 30 min after the addition of ATP (to 1 mM), GTP (to 0.1 mM), sodium phosphoenol pyruvate (to 5–10 mM), and pyruvate kinase (to 26–30 $\mu\text{g}/\text{ml}$). The preincubated extract (S10) was dialyzed overnight at 4 C against three 1-liter changes of buffer B and was then clarified by centrifugation at $10,000 \times g$ for 10 min and stored in small samples at -70°C .

Ribosomes and high-speed supernatants (S100) were prepared from preincubated S10 by centrifugation at 50,000 rpm for 3 h. The upper two-thirds of the supernatants was dialyzed against buffer B and the protein content was determined by the method of Lowry. The ribosomal pellet was resuspended in buffer B and the suspension was clarified by centrifugation at $10,000 \times g$ for 10 min. An optical density at 260 nm (OD_{260}) reading was obtained for the various cell fractions. Ribosomes and S100 were stored in small samples at -70°C .

Incorporation of amino acids in vitro. Cell-free extracts were mixed with an assay mixture that contains: 100 mM Tris-hydrochloride, pH 7.4; 6 mM magnesium acetate; 50 mM ammonium chloride; 6 mM 2-mercaptoethanol; 8.58 mM sodium phosphoenol pyruvate; 26–30 μg of pyruvate kinase per ml; 3×10^{-5} M GTP; 10^{-3} M ATP; 0.1 mM unlabeled amino acids; and 30 to 50 μCi of ^{35}S -methionine per ml or 3 to 5 μCi of ^{14}C -amino acid mixture per ml. Inhibitors, when used, were added prior to addition of the cell-free extract and radioactive amino acids.

The reaction was begun by the addition of the radioactive amino acid(s) and incubated at 37 C for 30 or 60 min. The incorporation of radioactive amino acids into protein was assayed by removing 30- to 50- μl samples at various times and precipitating with cold 10% trichloroacetic acid. The samples were heated at 90 C for 15 min, filtered on membrane filters (Millipore Corp.), and washed with cold 5% acid for determination of hot acid-precipitable radioactivity. The counting efficiency of ^{35}S -methionine is about 98%.

Acrylamide gel electrophoresis. Samples from in vitro reaction mixtures were precipitated by the addition of an equal volume of 10% trichloroacetic acid and washed with 1% acid and cold acetone. The

samples were dissociated and solubilized by the addition of 0.2 ml of solubilizing buffer. ^3H -amino acid-labeled, purified VSV was added, and the sample was boiled for 1 to 2 min. Sucrose was added to a final concentration of 10% and the sample was then layered on 18-cm 7.5% polyacrylamide-SDS gels. Electrophoresis and fractionation of the gels was as previously described (14, 18).

Sucrose density centrifugation of polysomes. Sodium deoxycholate (DOC) was added to in vitro reaction mixtures to a final concentration of 1%, and the samples were incubated at 4 C for 5 min. They were then layered on 11.4-ml, 7 to 47% (wt/wt) sucrose gradients in RSB and centrifuged at 4 C in the SW41 rotor at 37,000 rpm for 3.5 h. Fractions (0.4 ml) were collected and assayed for absorbance at 260 nm and hot acid-precipitable radioactivity.

The same procedure was used in analyzing ^3H -uridine-labeled crude cytoplasmic extracts, cytoplasmic supernatant and cytoplasmic pellets except that they were layered on 7 to 52% (wt/wt) sucrose gradients in RSB and centrifuged in the SW 27 rotor at 16,000 rpm for 16 to 17 h. Fractions (1.0 ml) were collected and assayed for absorbance at 260 nm and cold acid-precipitable radioactivity.

RESULTS

Characterization of the system. Incorporation of ^{35}S -methionine or a ^{14}C -amino acid mixture into hot acid-precipitable material, as directed by a crude, VSV-infected cytoplasmic extract, is linear for about 10 to 20 min and continues for approximately 30 to 40 min (Fig. 1). Incorporation is dependent upon the cytoplasmic extract, an energy-generating system, and is optimal at 6 mM Mg^{2+} and 50 mM NH_4^+ . As shown in Table 1, incorporation of amino acids by a crude cytoplasmic extract is decreased by inhibitors of mammalian protein synthesis.

Decrease of polysomes with time of incubation. In the experiment shown in Fig. 2, VSV-specific crude cytoplasmic extracts were incubated in the presence of ^{35}S -methionine for 10, 20, and 40 min, and subsequently the extracts were layered on sucrose gradients for an analysis of polysomes and nascent and released polypeptides. Initially, (10-min incubation, Fig. 2B) there is a large polysome region, as measured by OD_{260} , and a significant amount of nascent peptide associated with it. However, with increasing time of incubation the polysomes shift to a smaller size and concomitantly the nascent peptides also shift to smaller sized polysome regions and then are released to the top of the gradient (Fig. 2, Table 2). Examination of the material released, after a 60-min incubation period, on 7.5% SDS-acrylamide gels showed that all the VSV structural proteins (except for protein G) were synthesized and

FIG. 1. Kinetics of incorporation of ^{35}S -methionine in an in vitro system directed by a crude cytoplasmic extract from VSV-infected HeLa cells. A 0.3-ml sample of a crude cytoplasmic extract was mixed with 0.15 ml of a mixture containing all the requirements for protein synthesis. The reaction was begun by the addition of 15 μl of ^{35}S -methionine (830 $\mu\text{Ci}/\text{ml}$, 100 Ci/mmol). Incubation was for 60 min at 37 C. Fifty- μl samples were removed at various times and assayed for hot acid-precipitable radioactivity. Symbols: O, complete system; ●, minus extract.

released from mRNA in this in vitro system (data not shown).

Characterization of in vitro products on gels. VSV-specific crude cytoplasmic extracts were incubated in the presence of ^{35}S -methionine and the other requirements for in vitro protein synthesis for 10 and 30 min. The samples were then prepared for electrophoretic analysis and run on 7.5% SDS-acrylamide gels along with ^3H -amino acid-labeled purified VSV. Four VSV structural proteins and a number of minor viral-specified proteins are synthesized in the in vitro system (Fig. 3A-C). A striking observation is that little glycoprotein is made (Fig. 3A,B). Furthermore, when compared to the ratio of structural proteins found in the virion (Fig. 3C) there is a significantly larger amount of proteins L and NS synthesized in vitro. Both long- and short-term labeling of

TABLE 1. *Effect of inhibitors of protein synthesis on incorporation of ^{35}S -methionine directed by a crude cytoplasmic extract^a*

Conditions	^{35}S -methionine (counts/min)
Complete	35,300
– Extract	877
– Unlabeled amino acids	29,203
– ATP, GTP, PK, PEP	3,249
– PK, PEP	16,213
Complete	29,777
+ 6.6 μg of RNase (pancreatic)	412
+ 6.6 μg of DNase	25,045
Complete	19,768
+ 0.25% DOC	0
+ 5.6 μg of RNase	54
Complete	8,449
+ 10^{-5} M ATA	7,459
+ 3.3×10^{-5} M ATA	6,532
+ 10^{-4} M ATA	5,449
Complete	52,404
+ 2.67×10^{-5} M pactamycin	5,155
+ 2.67×10^{-6} M pactamycin	31,873
+ 2.67×10^{-7} M pactamycin	56,980
Complete	9,934
+ 9×10^{-4} M chloramphenicol	8,183
+ 0.78×10^{-4} M cycloheximide	7,315
+ 3.91×10^{-4} M cycloheximide	7,090
+ 7.81×10^{-4} M cycloheximide	5,189

^a The protein-synthesizing system was as described previously (Fig. 1, Materials and Methods). The inhibitor was added prior to the addition of the crude cytoplasmic extract. The reaction was begun by the addition of ^{35}S -methionine. Samples were assayed for hot acid-precipitable radioactivity (Materials and Methods). Zero-time samples were subtracted from all 60-min values.

VSV-infected cells also show an increased amount of protein NS compared with the virion, but very little protein L (5, 14).

Although the products synthesized at 10 and 30 min appear to be the same, there is a change in the ratio of viral structural proteins synthesized at 10 min compared with 30 min. During longer labeling times proteins N and M increase compared with proteins L and NS. This change in ratio does not occur during labeling for short (9 min) and long periods (90 min) in vivo (data not shown).

Fractionation of the system. Most of the protein-synthesizing activity of extracts from poliovirus-infected HeLa cells has been shown to be sedimentable at $20,000 \times g$ (17). However, this was not the case with extracts from type 5 adenovirus-infected KB cells (27). We also wanted to examine the protein-synthesizing activity of a fractionated VSV-infected HeLa cell system.

At 2-h postinfection, ^3H -uridine was added to

VSV-infected HeLa cells. At 4.5 h postinfection a crude cytoplasmic extract was prepared and this was fractionated by sedimentation at $20,000 \times g$ for 30 min into a pellet and a supernatant fluid. The crude cytoplasmic extract, cytoplasmic pellet, and cytoplasmic supernatant were incubated with DOC and

FIG. 2. Sucrose gradient centrifugation analysis of polysomes during various periods of *in vitro* protein synthesis directed by a crude cytoplasmic extract. Reaction mixtures were prepared by the addition of 0.3 ml of a crude cytoplasmic extract, 0.15 ml of a protein-synthesizing mixture, and 15 μl of ^{35}S -methionine (1,000 $\mu\text{Ci}/\text{ml}$, 128.9 Ci/mmol). Incubation was at 37 C for 10, 20, or 40 min. The reactions were stopped by the addition of DOC to a final concentration of 1% and the samples were further incubated at 4 C for 5 min. Samples were layered on 7 to 47% (wt/wt) sucrose gradients in RSB and centrifuged in the SW 41 rotor. The absorbance at 260 nm was continuously monitored by a Gilford recording spectrophotometer, and subsequently one drop of 0.2% bovine serum albumin and 1 ml of cold 10% trichloroacetic acid was added to each fraction collected. The samples were heated at 90 C for 15 min and filtered through membrane filters (0.45 μm pore size; Millipore Corp.). Hot acid-precipitable radioactivity was counted. As a control a crude cytoplasmic extract, which was not incubated at 37 C, was layered on a sucrose gradient, centrifuged, and processed as above. Symbols: solid line, optical density; O, counts per minute of ^{35}S -methionine. Monosomes are present in panel A fractions 16 to 18; panel B, 15 to 17; panel C, 16 to 18; panel D, 16 to 18. A, control, zero-minute incubation; B, 10-min incubation; C, 20-min incubation; D, 40-min incubation.

TABLE 2. Kinetics of polysome run-off and release of nascent peptides during *in vitro* protein synthesis^a

Incubation time (min)	Polysome region	Released protein	Total
10	68,569	26,948	108,501
20	87,959	69,944	199,806
40	56,018	88,719	202,917
60	19,210	141,084	201,767

^a The ³⁵S-methionine hot acid-precipitable counts in the gradients (see Fig. 2) were totaled. Fractions 1 to 14 were defined as being the polysome region and fractions 23 to 30 contained released protein, and the hot acid-precipitable counts in the above regions were totaled.

were then layered on 7 to 52% sucrose gradients in RSB. Polysomes were present in both the cytoplasmic pellet and the cytoplasmic supernatant, although the absolute amounts present in the two fractions were variable in different preparations. Furthermore, the cytoplasmic supernatant contained most of the monosomes and ribosomal subunits (data not shown).

When the protein-synthesizing activity of the three fractions was tested, we found that the activity of both the cytoplasmic pellet and the cytoplasmic supernatant was considerably reduced compared with the activity of the crude cytoplasmic extract (Fig. 4). A reconstituted extract containing a 1:1 ratio of cytoplasmic pellet and cytoplasmic supernatant was still not as active as the unfractionated system.

We next examined the products synthesized by the different fractions on polyacrylamide gels. The various extracts (including a ribosomal pellet obtained from the cytoplasmic supernatant) were incubated at 37°C for 30 min in the presence of ³⁵S-methionine. Figure 5 shows that both the cytoplasmic supernatant and the ribosomal pellet fractions (Fig. 5B and C) directed the synthesis of structural proteins N, NS, and M and a very significant amount of protein L. However, very little, if any, glycoprotein was synthesized. Synthesis of viral structural proteins L, N, NS, and M by the cytoplasmic pellet (Fig. 5A) was significantly reduced, but compared to the crude cytoplasmic extract (Fig. 3B), the cytoplasmic supernatant (Fig. 5B) or the ribosomal pellet (Fig. 5C), a relatively greater amount of the glycoprotein (G) was synthesized. Each of the fractions also appeared to synthesize a number of minor viral proteins.

Stimulation of the cytoplasmic pellet. Since the cytoplasmic pellet does contain a significant amount of polysomes, it is surprising that this fraction does not efficiently direct the incorpo-

FIG. 3. Analysis by SDS-acrylamide gel electrophoresis of the products synthesized by a crude cytoplasmic extract. A 0.2-ml sample of a crude cytoplasmic extract was incubated at 37°C with 0.1 ml of a protein-synthesizing mixture and 8 μ liters of ³⁵S-methionine (1,000 μ Ci/ml) (performed in duplicate). Incubation was for 10 or 30 min and the reaction was stopped by the addition of 0.3 ml 10% trichloroacetic acid. The samples were processed for electrophoresis on 7.5% SDS-acrylamide gels. Electrophoresis and fractionation of the gels have also been described. ³H-amino acid-labeled purified VSV was run on a separate gel. A, 10-min incubation; B, 30-min incubation; C, purified VSV.

ration of amino acids into viral-specific products. It is possible that factors are missing from the cytoplasmic pellet which restrict its ability

FIG. 4. Kinetics of *in vitro* incorporation of ^{35}S -methionine directed by extracts from a fractionated system. A 0.2-ml extract was mixed with 0.1 ml of a mixture containing all the requirements for protein synthesis. The reaction was begun by the addition of 10 μl of ^{35}S -methionine (940 $\mu\text{Ci/ml}$, 71.7 Ci/mmol) and incubation was for 40 min at 37 C. Thirty-microliter samples were removed at the indicated times and processed as described. The cytoplasmic supernatant fraction and cytoplasmic pellet were mixed in a 1:1 ratio (by volume) prior to incubation. Symbols: $\circ$, crude cytoplasmic extract (29.5 OD_{260} U per ml); $\bullet$, cytoplasmic supernatant (30.8 OD_{260} U per ml); Δ , cytoplasmic pellet (12.3 OD_{260} U per ml); $\blacktriangle$, cytoplasmic supernatant: cytoplasmic pellet (1:1).

to direct protein synthesis. We investigated this possibility by preparing preincubated extracts (S10) from uninfected HeLa and L cells. These extracts contain all the factors required for protein synthesis, but contain little endogenous protein-synthesizing activity (Fig. 6 and Table 3). The addition of S10 from either uninfected HeLa or L cells to the cytoplasmic pellet results in a 20- to 30-fold stimulation of incorporation of ^{35}S -methionine into hot acid-precipitable material (Fig. 6, Table 3), and the stimulation is dependent upon the amount of S10 added. The addition of rabbit liver tRNA stimulated incorporation twofold above that observed by HeLa S10 alone (not when L-cell S10 is used). However, when only rabbit liver tRNA is added to the cytoplasmic pellet there is no stimula-

tion. Preincubated HeLa or L-cell S10 does not stimulate incorporation of ^{35}S -methionine by crude cytoplasmic extracts or by the cytoplasmic supernatant (Table 3), nor does it change the pattern of products synthesized by these extracts.

We analyzed the products synthesized by the

FIG. 5. Analysis by SDS-acrylamide gel electrophoresis of the products synthesized by the cytoplasmic pellet, cytoplasmic supernatant fraction, and the ribosomal pellet. Reaction mixtures (as described in Fig. 3) were incubated at 37 C for 30 min in the presence of ^{35}S -methionine. The samples were prepared for electrophoresis on 7.5% SDS-acrylamide gels (described in Fig. 3). Note the different scales used in each panel. A, cytoplasmic pellet; B, cytoplasmic supernatant fraction; C, ribosomal pellet.

FIG. 6. Stimulation of ^{35}S -methionine incorporation by addition of uninfected HeLa S10 extracts to a VSV-infected cytoplasmic pellet. Varying amounts of HeLa S10 (26.8 OD_{260} U per ml) were added to a protein-synthesizing system containing 0.150 ml of cytoplasmic pellet. The total reaction volume was 0.300 ml. In some reactions, 165 μg of rabbit liver tRNA was added per ml. The reaction was begun by the addition of 10 μl of ^{35}S -methionine (760 $\mu\text{Ci/ml}$, 86 Ci/mmol) and incubation was at 37°C for 40 min. Forty-microliter samples were withdrawn at various times and processed. Symbols: ○, cytoplasmic pellet; ●, cytoplasmic pellet plus 40 μl of S10; △, cytoplasmic pellet plus 80 μl of S10; ▲, cytoplasmic pellet plus 80 μl of S10 plus tRNA; □, 80 μl of S10 plus tRNA.

cytoplasmic pellet when stimulated by HeLa- and L-cell S10 extracts. All the viral structural proteins and a number of minor viral proteins are synthesized (Fig. 7). Most importantly, both HeLa- and L-cell S10 extracts stimulate synthesis of the glycoprotein (G) (i.e., a viral-specified protein of approximately the same molecular weight as G). However, we do not know if this protein is glycosylated.

The preincubated HeLa S10 was separated into a ribosomal fraction and a supernatant fraction (S100). Table 4 indicates that the ribosomal fraction apparently contains most of the stimulatory activity. We are now attempting to further characterize the stimulatory factor(s).

DISCUSSION

Crude cytoplasmic extracts from VSV-infected HeLa cells are capable of directing the incorporation of amino acids into hot acid-

precipitable material, and incorporation by this in vitro system is linear for 10 to 20 min and continues for 30 to 40 min. Completion and release of VSV-specific nascent proteins from ribosomes occurs in this system; however, the extent of release of completed protein has not been determined.

Analysis by acrylamide gel electrophoresis of the products synthesized by crude cytoplasmic extracts from VSV-infected HeLa cells indicates that primarily VSV-specific proteins are made. Of the five VSV structural proteins, mainly four are synthesized in vitro by the crude cytoplasmic extract. Synthesis of the glycoprotein (G) is drastically inhibited. One possible explanation for this observation is that the protein portion of G is synthesized, but glycosylation does not occur in vitro. Since the effect of carbohydrates on the migration of glycoproteins in SDS-acrylamide gels is unknown, it is possible that the nonglycosylated protein is migrating to another part of the gel and is therefore undetected. Siebert et al. (19) found that in a cell-free lysate from *Escherichia coli*, RNA from avian myeloblastosis virus directed the synthesis of at least four proteins which had the same molecular weight as viral structural proteins. However, no product corresponding in molecular weight to one of the two viral glycoproteins was synthesized in vitro.

Our results do not reveal the presence of a significant amount of an additional viral protein which might correspond to nonglycosylated protein G. Also, both uninfected HeLa- and L-cell S10 stimulate the synthesis of protein G in a VSV-infected cytoplasmic pellet-directed system and this G protein peak comigrates in acrylamide gels with the G protein of purified VSV (Fig. 7). Therefore, it does not appear likely that the apparent inhibition of synthesis of protein G results from an anomalous migration of nonglycosylated protein G.

A second possibility is that the glycoprotein (or its protein portion) is synthesized as a large precursor polypeptide molecule which is not cleaved in vitro. Indirect evidence supporting this hypothesis is found in the in vitro system directed by the crude cytoplasmic extract, the cytoplasmic supernatant, or the ribosomal pellet. These systems synthesize a significantly greater amount of protein L (relative to the other virion structural proteins) than made in vivo or found in the virion. Since L is a very large protein of molecular weight 175,000, it is conceivable that the accumulation of excess amounts of this protein in vitro is the result of a lack of cleavage or processing of this large protein into smaller virion proteins. In experi-

TABLE 3. *Effect of uninfected, preincubated HeLa or L S10 extracts on incorporation of ^{35}S -methionine by VSV-infected extracts^a*

Extract	Addition	^{35}S -methionine (counts/min)
Cytoplasmic pellet	None	1,504
Cytoplasmic pellet	80 μ liters of HeLa S10	64,385
Cytoplasmic pellet	80 μ liters of HeLa S10 + tRNA	119,274
None	80 μ liters of HeLa S10 + tRNA	2,385
Cytoplasmic pellet	None	4,356
Cytoplasmic pellet	80 μ liters of L S10	134,350
Cytoplasmic pellet	80 μ liters of L S10 + tRNA	120,948
None	80 μ liters of L S10 + tRNA	3,251
Crude cytoplasmic extract	None	32,497
Crude cytoplasmic extract	tRNA	48,196
Crude cytoplasmic extract	80 μ liters of HeLa S10	39,758
Crude cytoplasmic extract	80 μ liters of HeLa S10 + tRNA	53,673
None	80 μ liters of HeLa S10 + tRNA	13,898
Cytoplasmic supernatant	None	7,533
Cytoplasmic supernatant	tRNA	6,455
Cytoplasmic supernatant	80 μ liters of HeLa S10	5,618
Cytoplasmic supernatant	80 μ liters of HeLa S10 + tRNA	6,526
None	80 μ liters of HeLa S10 + tRNA	4,501

^a Varying amounts of HeLa S10 (26.8 OD₂₆₀ U per ml) or L-cell S10 (26.8 OD₂₆₀ U per ml) were added to a protein-synthesizing system containing 0.150 ml of extract. The total reaction volume was 0.300 ml. In some cases, 165 μ g of rabbit liver tRNA was added per ml. The reaction was begun by the addition of 10 or 15 μ liters of ^{35}S -methionine (760 μ Ci/ml, 86 Ci/mmol). Incubation was at 37 C for 40 min and 40- μ liter samples were removed and processed as described. In each case, zero-time samples were subtracted from the 40-min values.

ments with protease inhibitors, which cause the accumulation of large precursor polypeptides in poliovirus-infected cells (12, 20) and in Sindbis virus-infected cells (15), we have found a slight accumulation of large proteins within the molecular weight range of protein L in both VSV-infected cells and in the *in vitro* system (unpublished observation).

Even though the amounts of L protein made *in vitro* are greater than the relative amounts made *in vivo* or found in infectious virions, none of our preliminary studies with the protease inhibitors were definitive. We are presently comparing tryptic peptides of L protein with those of G, N, NS, and M proteins to directly establish whether or not the L protein is a precursor polypeptide molecule of one or more of these other VSV structural polypeptides.

A third possibility could involve a requirement of specific factors for translation of one or more of the VSV-specific mRNAs. For example, there is evidence which indicates that glycosylation of some proteins begins while the protein is still associated with the polysomes (13, 22, S. Moyer, personal communication). Therefore, if the proper sugar transferases were not present in our cell-free extracts or if the conditions used for protein synthesis were not optimal for transferase activity, translation of the glycoprotein mRNA may be inhibited if translation is some-

how controlled by the addition of sugar residues during protein synthesis.

In support of this latter hypothesis we have apparently found that a factor(s) necessary for translation of VSV-specific mRNAs is missing or is present in reduced amounts in the VSV-infected cytoplasmic fractions. The addition of preincubated extracts from uninfected HeLa or L cells (S10) or HeLa ribosomes to the cytoplasmic pellet stimulates incorporation of ^{35}S -methionine by 30- to 60-fold, whereas incorporation of amino acids directed by the crude cytoplasmic extract or the cytoplasmic supernatant is not stimulated by S10. Furthermore, the products synthesized by a stimulated cytoplasmic pellet system include sizeable amounts of protein G and protein N as well as the other virion proteins (Fig. 7). We are now determining whether protein G synthesized *in vitro* is the same as that synthesized *in vivo* by comparing their tryptic peptide maps. In addition, work is in progress to obtain a cytoplasmic pellet stimulated *in vitro* system which can incorporate radiolabeled sugars into protein G.

The reason(s) why the crude cytoplasmic extract or the cytoplasmic supernatant is not stimulated by S10 extracts is not apparent, but this finding does raise the possibility that these fractions might contain an inhibitory factor which prevents translation of the G

mRNA. It might be possible that these two fractions contain larger amounts of this inhibitor than is contained in the cytoplasmic pellet and whatever is present in the S10 which stimulates translation of the G mRNA in the cytoplasmic pellet might be insufficient to overcome the inhibitor in the other two cytoplasmic fractions. We are now looking for the existence of such an "inhibitor" in crude cytoplasm and in cytoplasmic supernatant fractions.

We have also found that fractionation of the crude cytoplasmic extract into a cytoplasmic pellet and a cytoplasmic supernatant fraction results in a significant reduction of the protein-synthesizing activity of both these fractions as compared to the unfractionated system, al-

TABLE 4. Effect of uninfected, preincubated HeLa ribosomal fractions or HeLa S100 on the protein-synthesizing activity of a VSV-infected cytoplasmic pellet^a

Extract	Addition	³⁵ S-methionine (counts/min)
Cytoplasmic pellet	None	
Cytoplasmic pellet	20 μ liters of HeLa ribosomes	18,713
Cytoplasmic pellet	40 μ liters of HeLa ribosomes	39,395
Cytoplasmic pellet	80 μ liters of HeLa ribosomes	65,088
Cytoplasmic pellet	80 μ liters of HeLa ribosomes + tRNA	104,247
None	80 μ liters of HeLa ribosomes + tRNA	1,252
Cytoplasmic pellet	None	
Cytoplasmic pellet	108 μ g of HeLa S100	1,012
Cytoplasmic pellet	215 μ g of HeLa S100	6,832
Cytoplasmic pellet	215 μ g of HeLa S100 + tRNA	5,592
None	215 μ g of HeLa S100 + tRNA	1,366

^a Varying amounts of HeLa ribosomes (23.1 OD₂₆₀ U per ml) or HeLa S100 (2.69 mg/ml) were added to a protein-synthesizing system containing 0.150 ml cytoplasmic pellet (same extract as used in experiments of Table 3). The total reaction volume was 0.300 ml. In some cases, 165 μ g of rabbit liver tRNA was added per ml. The reaction was begun by the addition of 10 μ liters of ³⁵S-methionine (940 μ Ci/ml, 71.7 Ci/mmol). Incubation was at 37 C for 40 min. 40- μ liter samples were removed and processed as previously described. In each case, zero-time samples were subtracted from the 40-min values.

FIG. 7. Analysis of the products synthesized by the cytoplasmic pellet when stimulated by HeLa or L S10. Reaction mixtures in the amount of 0.300 ml containing 0.150 ml of cytoplasmic pellet, 0.080 ml of HeLa or L S10, 165 μ g of rabbit liver tRNA per ml (only added in the HeLa-S10-stimulated system) plus 0.010 ml of ³⁵S-methionine (760 μ Ci/ml, 86 Ci/mmol) and all the other requirements for protein synthesis were incubated at 37 C for 30 min. The reaction was stopped by the addition of an equal volume of 10% trichloroacetic acid. The samples were processed for electrophoresis on 7.5% SDS-acrylamide gels. A, HeLa-S10 stimulated, B, L-S10 stimulated.

though both fractions contain significant amounts of VSV-specific polysomes. The products synthesized by the cytoplasmic supernatant fraction and the cytoplasmic pellet differ. The cytoplasmic supernatant fraction (and a ribosomal pellet obtained from the cytoplasmic supernatant fraction) synthesizes a significant amount of proteins L, N, NS, and M, but very little, if any, protein G. The ratio of structural proteins synthesized by the cytoplasmic pellet indicates that protein N is reduced relative to the amount of N synthesized by the crude cytoplasmic extract. Also, this fraction seems to synthesize small amounts of protein G (Fig. 5A). This might suggest that there has been a fractionation of some VSV-specific mRNAs between the cytoplasmic supernatant fraction and the cytoplasmic pellet. A more detailed analysis of the fractionation of this system into membrane-bound and free polysomes and an analysis of ³H-uridine VSV-specific mRNA contained in each fraction might provide answers about the cellular sites of VSV protein synthesis.

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