JOURNAL OF VIROLOGY, Sept. 1974, p. 652-663
Vol. 14, No. 3

Effects of Different RNAs and Components of the Cell-Free System on In Vitro Synthesis of Sindbis Viral Proteins

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Received for publication 29 April 1974

Cell-free extracts from Krebs ascites cells and rabbit reticulocytes synthesized a variety of viral-specific proteins when programmed with several different kinds of Sindbis viral RNAs. The RNAs included purified virion RNA (42*S*) and two species (26*S* and "33*S*") of purified intracellular viral messenger RNAs from viral-infected BHK cells. Proteins formed in vitro were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, rate-zonal centrifugation in urea-sucrose gradients, two-dimensional tryptic peptide fingerprints, and immunoprecipitation with rabbit anti-Sindbis virus serum. The only major identifiable protein formed in vitro was viral capsid, but the relative amount of capsid produced was determined by the mRNA, the source of cell-free extract, and the components of the cell-free system. Virion RNA directed synthesis of larger-molecular-weight proteins than did intracellular viral RNAs, and some of this protein was distinct from that formed by the smaller viral RNAs. Indirect evidence is presented for in vitro synthesis of viral envelope proteins.

We recently reported that cell-free extracts from both Krebs ascites cells and rabbit reticulocytes formed Sindbis virus capsid protein when supplemented with preparations of partially purified Sindbis viral mRNA (5). Although the viral capsid was the predominant polypeptide synthesized in vitro, three additional discrete polypeptides were often detected in autoradiograms of sodium dodecyl sulfate (SDS)-polyacrylamide gel electropherograms. Tryptic peptide fingerprints of these isolated polypeptides showed that they all contained capsid sequences. The kinetics of labeling of these proteins and capsid indicated that the four proteins appeared sequentially in order of their size, with the smallest appearing in less than 5 min of incubation and the larger at 10 min. By 15 min no new discrete proteins appeared, and about four to five times more capsid was present than the other three proteins combined.

On the basis of these data and assuming a single site for initiation of translation, we postulated that there were a few discrete places along the viral mRNA where translation was slowed or halted so that discrete peptides were released. One of these points coincided with the carboxyl-terminal position of the viral capsid cistron, thereby leading to a polypeptide indistinguishable from the authentic capsid of the virion. This mechanism for formation of Sindbis capsid is quite distinct from that proposed to account for virion protein production in Sindbis virus-infected cells. Results from experiments with infected cells led to the hypothesis that the virion proteins originated by post-translational cleavage of a large precursor polypeptide that was encoded by a polycistronic mRNA (15, 17). According to this model, the mRNA initiated translation only at the 5′ terminus of the nucleic acid, and capsid and envelope proteins of the virion arose after several stages of proteolysis, some of them occurring while the polypeptide was being translated. Not all the data obtained from studies with Sindbis virus supported this model, but it was analogous to a mechanism established for the formation of picornavirus proteins such as polio and encephalomyocarditis viruses (4, 11, 12, 22). The strongest support for this in vivo mechanism of Sindbis virus protein synthesis was the observation that the temperature-sensitive mutant ts-2 of Sindbis virus accumulates a large polypeptide at nonpermissive temperature (19) and that this protein contained the sequences of the three virion proteins (20).

In our initial studies of the in vitro synthesis of Sindbis viral proteins, we failed to obtain clear evidence for either post-translational proteolysis or even for translation of discrete poly-

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peptides much larger than capsid. We have further examined the in vitro system, and in this report we describe the alterations in the protein patterns that result when different species of viral RNAs are added to the cell-free system. We also show how changing the concentrations of components in the rabbit reticulocyte cell-free system can influence the synthesis of both viral-specific and endogenous proteins. In the following paper (6), the in vitro synthesis of protein directed by RNA from a temperature-sensitive Sindbis virus mutant is described.

MATERIALS AND METHODS

Isolation of RNA. The preparation and partial purification of RNA from virions and from viral-infected BHK cells have been described in detail (5). The procedure leads to a fivefold purification of RNA extracted from cells (Table 1). The fraction eluted from the oligo(dT) cellulose column (biological activity = 4.12; see Table 1 for definition of biological activity) was equivalent to RNA from virions (biological activity = 4.8) in terms of its ability to stimulate protein synthesis in a cell-free extract from Krebs ascites cells. Different preparations of viral messenger RNAs varied with regard to their ability to stimulate in vitro protein synthesis in different cell-free extracts, but virion RNA and partially purified intracellular viral-specific RNA were roughly equivalent, on a mass basis, in stimulating in vitro protein synthesis (see below and Table 2). The 26*S* and "33*S*" intracellular viral-specific RNAs were isolated by electrophoresis in a 1.8% acrylamide. 0.5% agarose gel (23). Slices of the gel were eluted by shaking for 16 h at 23 C with 10 mM Tris-chloride, pH 7.4, 0.5 M LiCl. Those fractions containing ^{3}H counts per minute and with a mobility corresponding to 26*S* or "33*S*" were pooled and applied to the oligo(dT) cellulose column equilibrated with the same buffer. They were eluted with 10 mM Tris-chloride, pH 7.4, precipitated with ethanol, and resuspended in a small volume of water. The preparation of [^{14}C]RNA used for markers as 18*S* and 28*S* have been described (23).

Cell-free protein synthesis systems. The cell-free extract from reticulocytes, referred to as CFSII, was used here; it contains an S-100 supernatant fraction, salt-washed ribosomes, and the ribosomal salt-wash fraction. The relative amounts of the three fractions for 1 ml of reaction mixture were 0.5 ml, 0.12 ml, and 0.2 mg, respectively. Other details for preparation of these components and the extracts from Krebs ascites cells, as well as the materials for in vitro protein synthesis, were exactly as described previously (5).

Preparation and analysis of proteins made in vitro. The methods for isolating viral proteins made in vitro and their analysis on SDS-polyacrylamide gels were identical to those noted previously (5, 23). For the analysis by centrifugation in a sucrose gradient, the reduced alkylated samples (0.005 ml) were added to 0.025 ml of a solution consisting of 6 M urea and 0.5% SDS in a buffer (called TNE) of 0.1 M NaCl, 50 mM Tris-chloride, pH 7.4, and 1 mM EDTA. After 10 min at 37 C, 0.15 ml of TNE and 0.5% SDS were added, and samples were applied to 4.8 ml of 0 to 18% (wt/vol) sucrose gradient containing 2 M urea and 0.5% SDS in TNE. Centrifugation was at 38,000 rpm in the Spinco SW50 rotor at 20 C for 17 h. Each gradient was collected in 30 fractions, and 0.1 ml of each fraction was sampled for hot trichloroacetic acid-precipitable counts per minute.

Immunoprecipitation. To obtain the precipitins, an initial incubation period of 36 h at 4 C was carried out with mixtures containing 0.025 ml of rabbit antiserum, 0.025 ml of 1% SDS, 0.025 ml of M Tris-chloride, pH 7.4, 0.4 ml of 0.15 M NaCl, and 0.01 to 0.015 ml of in vitro reaction mixture carried out with Krebs ascites cell extract (the in vitro synthesis had been stopped by the addition of RNase). After the incubation with rabbit antiserum, to each sample was added 0.38 ml of 0.15 M NaCl, 0.025 ml of 1% SDS, 0.025 ml of M Tris-chloride, pH 7.4, and 0.07 ml of a preparation of goat anti-rabbit globulin (250 μg/ml) (Microbiological Associates, Bethesda, Md.). After 12 h at 4 C, the precipitins were collected by centrifugation and washed three times with cold 0.15 M NaCl.

TABLE 1. *Purification of Sindbis mRNA from infected BHK cells*

Fraction	[^{3}H]uridine counts/min/A_{260} units ($\times 10^4$)	Biological activity[a]
Cytoplasmic extract	22	1.68
Precipitated by $LiCl_2$	ND[b]	ND
Retained by oligo(dT) column	110	4.12
Not retained by oligo(dT) column	12	1.58

[a] Defined as the ratio of counts per minute incorporated by addition of mRNA to counts per minute in the absence of mRNA. The reaction was carried out with Krebs ascites extracts and contained 0.08 A_{260} units of RNA in a volume of 40 μliters that was incubated at 29 C for 60 min. With no added RNA, 3,000 counts/min of [^{35}S]protein was formed. Virion RNA had a biological activity of 4.8.

[b] Not done.

TABLE 2. *Stimulation of in vitro protein synthesis by different viral RNAs*

RNA species (μg added)[a]	Cell-free system	^{35}S incorporated[b] (counts/min)
None	Ascites	38,000
Virion (4.0)	Ascites	75,000
26*S* (2.9)	Ascites	124,000
"33*S*" (5.5)	Ascites	126,000
None	Reticulocyte CFS II	286,000
Virion (2.3)	Reticulocyte CFS II	566,000
26*S* (1.4)	Reticulocyte CFS II	430,000
"33*S*" (2.4)	Reticulocyte CFS II	571,000

[a] Total volume of reaction mixture was 25 μliters.

[b] Determined as hot trichloroacetic acid-precipitable counts per minute after 90 min (ascites) or 60 min (reticulocytes) at 29 C.

The precipitins were dried in vacuo and dissolved in 0.02 ml of a 2% SDS solution, and a sample was precipitated with trichloroacetic acid. Another sample was analyzed by electrophoresis in SDS-polyacrylamide gels. The antiserum used in these experiments was obtained from a rabbit injected with a purified sample of intact Sindbis virus that had been mixed with complete Freund adjuvant. Antibodies in this serum neutralize infectious virus and bind to infected cells that are actively producing virus (J. Symington, unpublished data).

Determination of radioactivity. [^{35}S]proteins and [^{3}H]nucleic acids were precipitated with trichloroacetic acid, collected on filters, and counted in a Tri-Carb scintillation counter. [^{35}S]methionine (60 Ci/mmol), [^{3}H]adenosine (30 Ci/mmol), [^{3}H]uridine (40 Ci/mmol), all from New England Nuclear, were used for labeling.

RESULTS

Effect of RNA species on in vitro synthesis of viral proteins. Sindbis virus contains a single strand of RNA of molecular weight (MW) 4.3×10^6 as its genome (7, 16, 21). This RNA is infectious (18), contains poly(A) sequences (9, 10), and is presumed to function as a messenger RNA in the infected cell (9, 14, 17). We isolated virion RNA and partially purified it by rate-zonal sedimentation in a sucrose gradient (Fig. 1); its *S* value was calculated to be 42*S*. Sindbis virus-infected BHK cells display several species of viral-specific RNA, but the predominant form has a sedimentation coefficient of 26*S* (16, 17, 21, 23). In our preparations, we routinely detected two major size classes of viral-specific intracellular RNA. One of these was identical to the 26*S* commonly observed, and the other had a molecular weight, calculated on its mobility in acrylamide gels, ranging from 2.3×10^6 to 3.0×10^6 (Fig. 2). These two species were purified and tested for their ability to direct viral protein synthesis. The relative proportions of 26*S* and the larger species, referred to here as "33*S*," varied among the different preparations, and some of our initial extracts from viral-infected cells contained about equal amounts of the 26*S* and "33*S*" species.

The 26*S*, "33*S*", and virion RNA stimulate the incorporation of [^{35}S]methionine into protein in both cell-free systems (Table 2). The relative effectiveness of the specific RNAs was not rigorously determined, but in the reticulocyte system, they were roughly equivalent based on the mass of RNA used. However, the pattern of proteins formed with the different RNAs was distinctive (Fig. 3 and 4). The purified 26*S* RNA directed synthesis primarily of polypeptides with molecular weights close to that of the virion capsid (MW = 30,000). In our previous published work on this system (5), we showed that band II of Fig. 3 is authentic capsid and noted that bands I and III have capsid se-

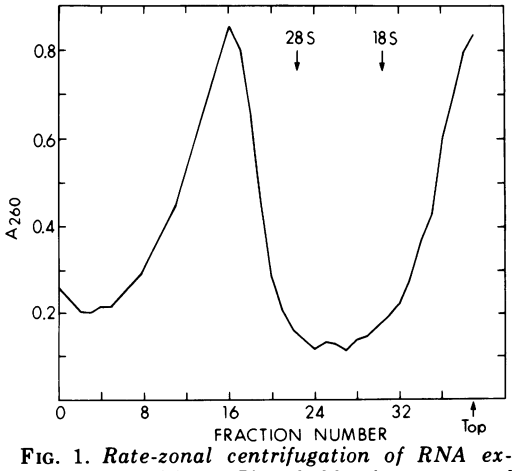

FIG. 1. *Rate-zonal centrifugation of RNA extracted from virions. Phenol-chloroform-extracted RNA, after precipitation with ethanol, was layered onto a 10 to 20% sucrose gradient (wt/wt) and centrifuged for 12 h at 20,000 rpm at 15 C in the Spinco rotor SW27. This amount of RNA came from a sample of virus containing a total of 10^{12} PFU.*

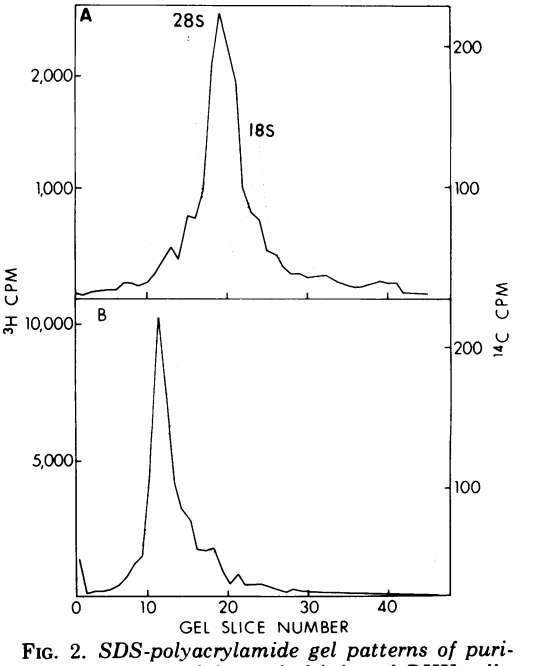

FIG. 2. *SDS-polyacrylamide gel patterns of purified RNAs extracted from viral-infected BHK cells. The gels contained 1.8% acrylamide and 0.5% agarose. (A) 26S fraction. (B) "33S" fraction. Symbols: —, ^{3}H; ...., ^{14}C from marker ribosomal RNAs.*

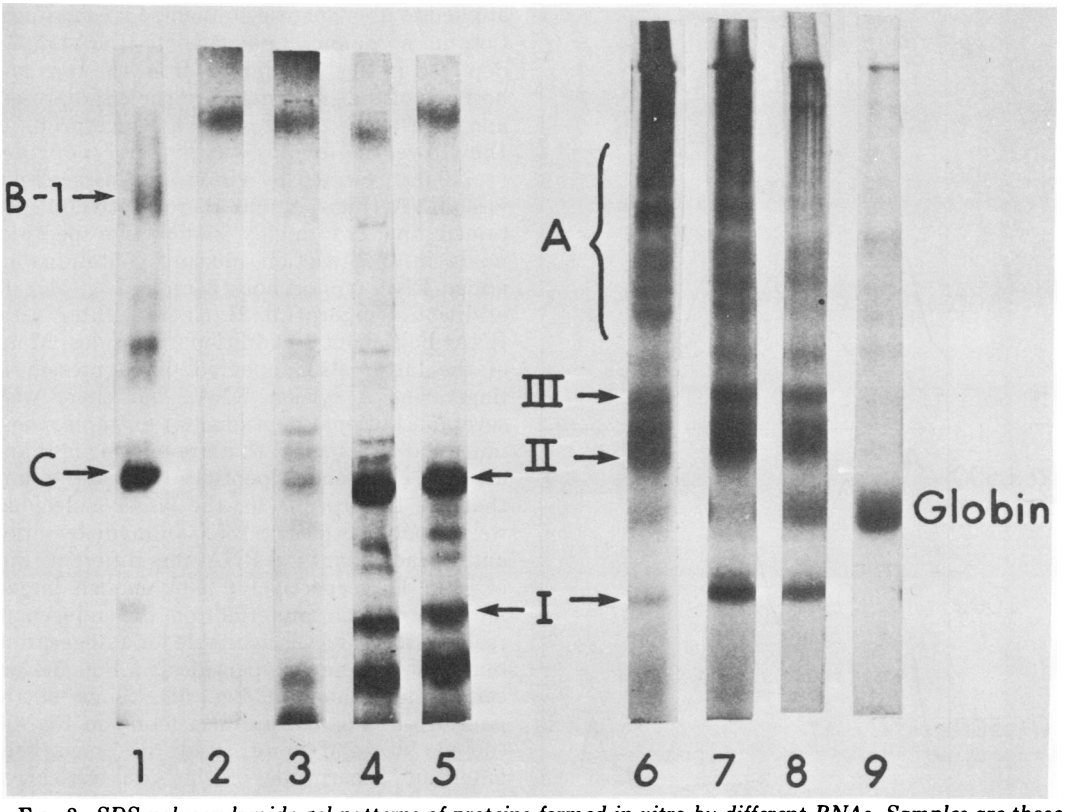

FIG. 3. *SDS-polyacrylamide gel patterns of proteins formed in vitro by different RNAs. Samples are those described in Table 2; 2–5 were from ascites cell-free extracts, and 6–9 were from a reticulocyte cell-free system (see ref. 5 for details of the incubation mixtures). Samples 3 and 6, virion RNA; 4 and 7, "33S" RNA; 5 and 8, 26S RNA; 2 and 9, no RNA added. Sample 1 was from BHK-infected cells labeled 5 h after infection for 30 min. B-1 refers to a protein containing viral envelope sequences; C refers to viral capsid. Equal amounts of the in vitro reaction mixtures (0.005 ml) after reduction and alkylation of proteins were added to gels.*

quences. The virion RNA (samples 3 and 6, Fig. 3) makes less of these capsid-like proteins and more of a larger-molecular-weight class of proteins, with considerable material remaining near the interface between the 5% acrylamide stacking gel and the 10% acrylamide running gel. The discrete proteins noted in region A of Fig. 3 may be endogenous proteins (see below). The radioactive material at the top of the gel in samples 6 to 8 (Fig. 3) cannot be resolved into discrete bands by using lower concentrations of acrylamide. We were unable to clearly identify as products of the in vitro reaction the two larger Sindbis virus proteins noted as B-1 or ts-2. These putative precursors of the virion proteins have been observed in Sindbis-infected cells and contain sequences of the virion proteins (20). The "33*S*" species functions as if it were a mixture of 26*S* and virion RNA, for it directs synthesis of both capsid material and larger-molecular-weight proteins (samples 4 and 7, Fig. 3). These observations are supported by an analysis of the proteins formed in vitro by using rate-zonal centrifugation in sucrose gradients containing urea and SDS (Fig. 4). The 26*S* RNA directs formation of proteins with a range of molecular weights less than 100,000, whereas virion RNA directs mostly the synthesis of proteins with molecular weights larger than 130,000. The "33*S*" RNA directs synthesis of both large and small proteins; the profile from the gradient of Fig. 4 (panel B) resembles a combination of the profiles obtained from reactions using the 26*S* and the virion RNAs. Included in Fig. 4 is the profile for a purified preparation of the Sindbis virus ts-2 protein. This polypeptide is composed of the three virion proteins, with a total estimated MW about 130,000, in covalent linkage (20). Its mobility in the sucrose-urea-SDS gradient closely resembles that of the marker *E. coli* beta-galactosidase subunit of MW = 135,000.

Tryptic peptide fingerprint analysis of protein formed in vitro by viral RNAs. The slab

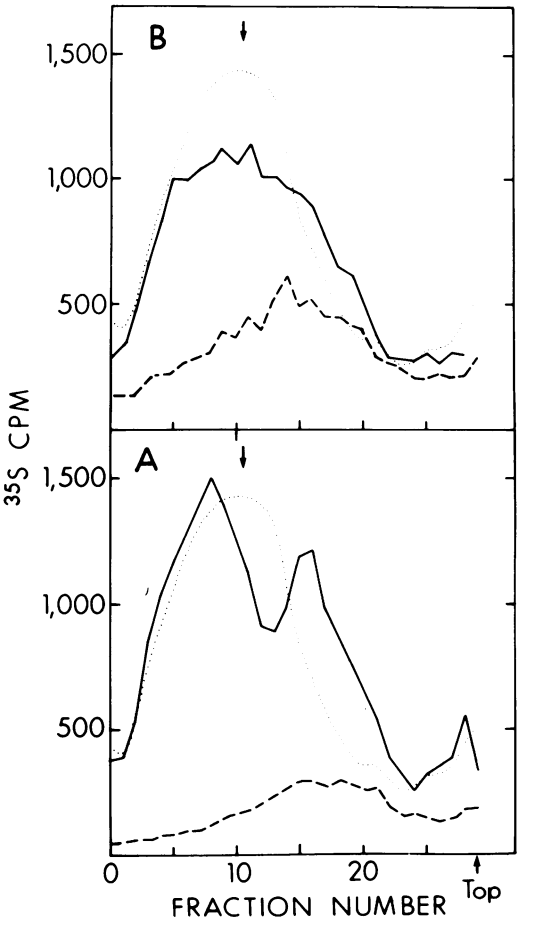

FIG. 4. *Rate-zonal centrifugation of protein formed in the reticulocyte system. Samples were obtained from reaction mixtures programmed with different viral RNAs after 60 min at 29 C. After denaturing the proteins by reduction and alkylation, samples were treated with urea and layered onto a 0 to 18% sucrose-urea gradient. Centrifugation was carried out in a SW50 Spinco rotor at 20 C for 38,000 rpm for 17 h. (A) ---, No viral RNA; —, virion RNA. (B) ---, 26S RNA; —, "33S" RNA. Samples were from those described in Fig. 3 and Table 2. The dotted line is the profile of a purified sample of [^{14}C]arginine-labeled ts-2 protein (700 counts/min in peak tube) centrifuged in a separate parallel gradient. The arrow is the peak fraction from a gradient containing β-galactosidase subunits; measured by A_{280}.*

gel protein patterns of Fig. 3 and the sucrose gradient analyses of Fig. 4 strongly suggest that the various species of viral RNAs were translated differently. Further evidence for this was found in tryptic peptide fingerprints of the ^{35}S-labeled proteins made in vitro. The polypeptides formed in the reaction mixtures were denatured by reduction and alkylation and applied to urea-sucrose gradients for centrifugation in a manner analogous to the samples depicted in Fig. 4. Samples from the tops and bottoms of the gradients were pooled, dialyzed, and digested with trypsin. The fingerprints of the lower-molecular-weight polypeptides (<110,000) formed by virion and intracellular viral RNA (Fig. 5A and B, respectively) contained the five highly radioactive peptides made in the reaction mixture containing no added RNA (endogenous sample, Fig. 5E). In addition, fingerprint B (intracellular viral RNA) had about 10 additional peptides. Many of the latter also appeared to be present in fingerprint A (virion RNA), but there were several additional peptides (for example, those noted "a" in panel A) that are missing in B and are not endogenous peptides. We also found that the fingerprints for the larger-molecular-weight polypeptides (>135,000) made by virion and intracellular viral RNA were different (Fig. 5C and D, respectively). Not enough larger-molecular-weight material from the endogenous reaction mixture was available for a fingerprint, but none of the polypeptides in Fig. 5C are easily identifiable in Fig. 5D. Some of the peptides in Fig. 5B are also found in Fig. 5D (noted "d") and those noted "d*" have been found in fingerprints of the viral capsid. A tryptic peptide fingerprint of ^{35}S-labeled B-1 protein that was isolated from Sindbis-infected BHK cells and contained the amino acid sequences of the Sindbis envelope proteins is shown in Fig. 5F. At this point we are unable to clearly assign peptides in the fingerprints of Fig. 5A–D to a viral envelope protein. In these studies, intracellular viral RNA was a preparation from the oligo(dT) column step (Table 1), and its electrophoretic profile showed it to contain both 26S and "33S" fractions in roughly equal amounts. Despite the complex assortment of tryptic peptides in the fingerprints of Fig. 5, the data are consistent with the hypothesis that the larger virion RNA is translated differently than the smaller intracellular viral RNAs.

Effect of the S-100 fraction and ribosomal salt-wash fraction from rabbit reticulocytes on in vitro protein synthesis. It is possible to vary the relative amounts of factors required for in vitro protein synthesis when one uses the partially fractionated extracts of rabbit reticulocytes (1). The system we used consisted of three fractions: a ribosomal salt wash, an S-100 supernatant fraction, and a ribosomal fraction. The salt wash is presumed to contain components important in the initiation of polypeptide formation, whereas the S-100 fraction contains tRNA species and soluble enzymes required for

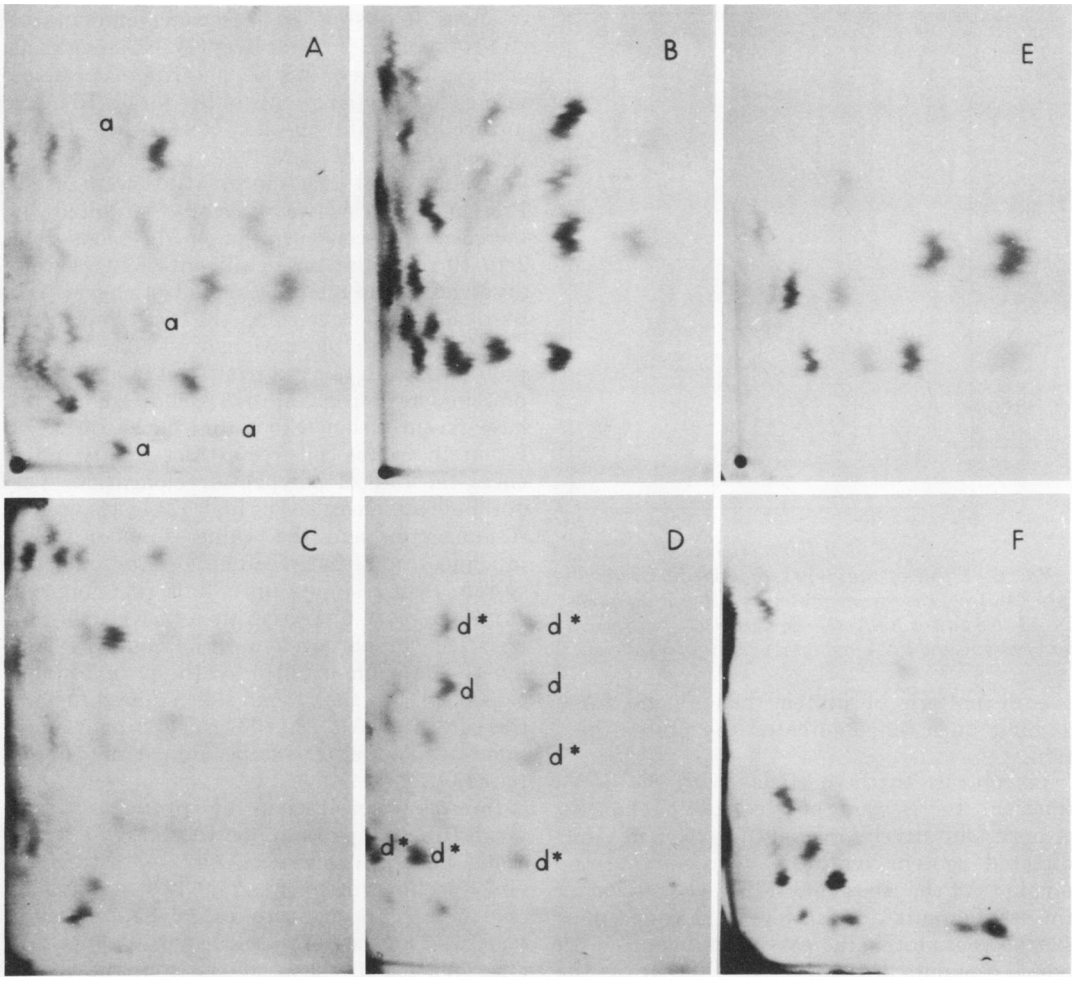

FIG. 5. *Autoradiograms of tryptic peptide fingerprints. Samples were collected from fractions 1 to 10 (>135,000 MW) and 15 to 25 (<110,000 MW) of urea-sucrose density gradients (similar to those in Fig. 4). (A) Small virion RNA, 5,000 counts/min; (B) small viral RNA, 14,000 counts/min; (C) large virion RNA, 2,000 counts/min; (D) large viral RNA, 3,500 counts/min; (E) small, no RNA added, 9,000 counts/min; (F) B-1 protein from infected-BHK cells. The counts per minute noted were from 0.02 ml of 0.5-ml sample of pooled, dialyzed, and digested fractions. Samples were applied in the lower-left corner of the papers; electrophoresis was in pH 3.5 buffer (upwards in the figure) followed by descending chromatography in butanol-acetic-acid-water (2:0.5:2.5) (to the right in the panels).*

elongation steps in protein synthesis. In our standard mixtures, one-half the total volume of the reaction solution was the S-100 fraction, and about 15% of the total volume was the salt-wash fraction. When we changed the amount of the S-100 fraction, the total amount of [^{35}S]methionine incorporated into protein changed by as much as three- to fourfold (Fig. 6). The increase in amount of synthesis was accompanied by a qualitative change in the pattern of proteins detected in both SDS-polyacrylamide gels (Fig. 7A) and in a rate zonal centrifugation in sucrose-urea-SDS gradients (Fig. 8). At low levels of S-100 fraction, more of the lower-molecular-weight proteins appeared. At the highest levels used, a considerable amount of the protein formed accumulated at the top of the 10% polyacrylamide gel (samples 3 and 4, Fig. 7A) and sedimented faster than the 135,000 MW marker in the sucrose gradient. When virion RNA was present, the increased amounts of S-100 fraction also led to more of the larger-molecular-weight material (data not shown). In the absence of added RNA (the endogenous reaction), increased amounts of S-100 fraction produced little or no qualitative changes in the

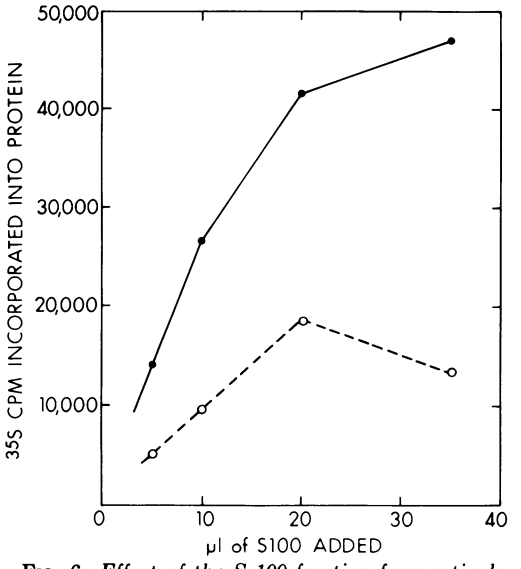

FIG. 6. *Effect of the S-100 fraction from reticulocyte cell-free system on protein synthesis. Symbols;* O....O, *No added RNA;* ●—●, *with 8 μg of intracellular viral-specific RNA in a total volume of 50 μliters.*

overall pattern of protein bands, and large amount of globin dominated the protein pattern.

In contrast to these effects with the S-100 fraction, there were no detectable changes, either qualitatively or quantitatively, in viral-directed protein synthesis upon varying the amounts of the ribosomal salt-wash fraction on the reaction mixture. However, the endogenous synthesis of protein increased significantly with added amounts of this fraction (Fig. 9). At the higher levels of this fraction, we detected a number of discrete protein bands in the SDS-polyacrylamide gel electropherograms (Fig. 7B, sample 2). Many of these latter bands comigrate with proteins observed in the reaction mixtures containing intracellular viral-specific and virion mRNAs, and it remains to be determined whether the higher-molecular-weight proteins noted in the preparations containing these RNAs (refer also to Fig. 3, region A of sample 6) are in fact viral-specific proteins. The stimulation by the ribosomal salt wash of endogenous synthesis may be the result of releasing from ribosomes endogenous messenger RNAs that subsequently can be more effectively translated in the CFS II reticulocyte system. We have noted previously (5) that addition of viral mRNA can inhibit endogenous synthesis (Fig. 9); but even under these conditions, viral-specific proteins can be detected (Fig. 7B, sample 4).

We also tested the response of the in vitro reticulocyte system to increasing amounts of tRNA prepared from rat liver (2). No significant changes occurred in the pattern of proteins formed in reactions with either virion RNA or intracellular viral-specific RNA when tRNA concentrations of 0 to 0.3 A_{260} units per 50 μliters of total reaction mixture were used. Protein synthesis was severely inhibited by increasing the concentration of Mg^{2+} ions from 2 to 10 mM, but no significant changes were observed in the relative amount of capsid protein produced. Increasing the KCl concentration from 80 to 160 mM had no effect on the protein pattern nor did it affect the amount of protein synthesized. Mathews and Osborn (13) have recently shown that the concentration of K^+ in an ascites cell-free extract greatly influences the rate of polypeptide elongation. The optimal rate measured at high K^+ levels was 35 residues/min, and this value is noted to be much less than that recorded for a reticulocyte system (300 residues/min). It is therefore not surprising that alteration in K^+ levels showed no effect in our system. From our previous kinetic data on in vitro synthesis of Sindbis capsid (5), less than 5 min was required to form the capsid (MW = 30,000), suggesting a minimal rate of about 50 residues/min at 29 C in the reticulocyte system.

Immunoprecipitation of products from viral-directed protein synthesis. One technique that is commonly used to distinguish viral-specific proteins in an in vitro system is to test the reaction mixture for polypeptides that react with antibodies raised against purified virions. We tested the products from the Krebs ascites cell-free system containing virion or intracellular viral-specific RNA with rabbit sera containing anti-Sindbis antibodies. Because of the low amount of viral antigen generated in vitro, we used a goat anti-rabbit serum to obtain a precipitate. There was a considerable amount of ^{35}S-labeled protein that precipitated in this procedure with a nonviral specific rabbit antiserum (Table 3, lines 4 and 6). Despite this high background, the reaction mixture that contained intracellular viral-specific mRNA showed a significant amount (a net of 4.7%) of viral-specific antigenic material (Table 3). The precipitates obtained with the specific and control antisera were examined by SDS-polyacrylamide gel electrophoresis and both showed a weak band corresponding to the capsid protein (Fig. 10). In addition, the precipitate from the specific antiserum contained material at the top of the gel that was not present in the precipitate formed with control antiserum. The [^{35}S]protein may represent peptide fragments

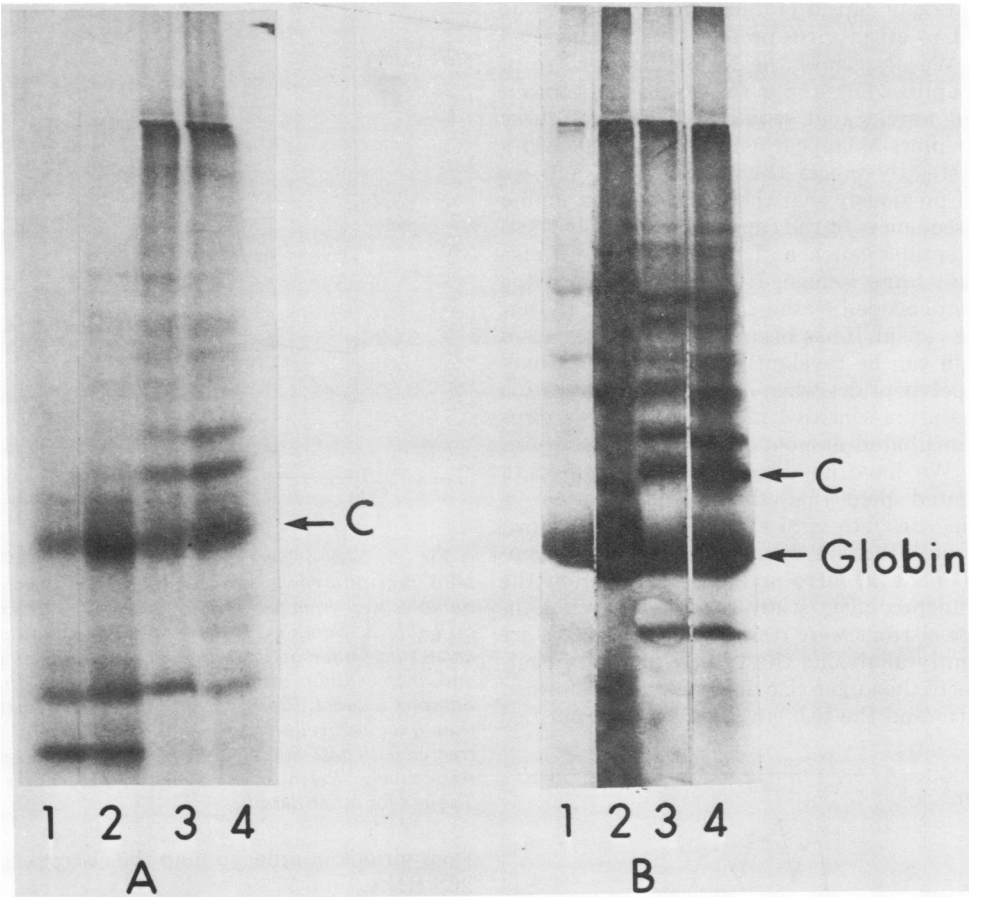

FIG. 7. *(A) SDS-polyacrylamide gel pattern of proteins formed in vitro with differing amounts of S-100 fraction. Samples 1, 2, 3, and 4 contained 5, 10, 20, and 35 μliters, respectively, of S-100 in a total volume of 50 μliters. Other components of the system were identical in concentrations in the different reaction mixtures. Intracellular viral-specific RNA (8 μg) was used. (B) SDS-polyacrylamide gel pattern of proteins formed in cell-free reticulocyte system containing varying amounts of ribosomal salt-wash fraction. Sample 1 and 2 had 0 and 3 μliters of salt-wash fraction, respectively, and no viral RNA; samples 3 and 4 contained 1.2 μg of intracellular viral-specific RNA and 0 and 3 μliters of salt-wash fraction, respectively. C refers to viral capsid.*

translated from the envelope cistrons, but possibly because they are part of membranous proteins they have a greater tendency to aggregate. We might anticipate that most of the anti-Sindbis virus antibodies are, in fact, directed against the envelope proteins of the virus. The antiserum used here does neutralize infectious Sindbis virus; however, it has not been tested for its ability to cross-react against viral-specific proteins.

The incorporation values of [^{35}S]methionine into protein in the reaction mixtures used for these analyses were the following: 8,000 counts/min with no added RNA; 13,000 counts/min with added virion RNA; and 25,000 counts/min with added intracellular viral-specific RNA. It is noteworthy that the excess amount of protein made in the ascites reaction mixture provided with virion RNA did not cross-react with viral antisera. This result suggests again that virion RNA is directing synthesis of nonvirion proteins.

DISCUSSION

Data reported in these studies provide some definitive information about the genetic composition of Sindbis viral RNAs. The size of the major species of RNA detected on polyribosomes of Sindbis virus-infected cells is 26*S*, and it was postulated that this RNA contains the three cistrons that code for the three virion proteins. We have confirmed that this RNA codes for virion capsid by demonstrating the

formation of capsid protein when 26S RNA was added to an in vitro protein-synthesizing system. We also show the formation in vitro of polypeptides larger than the capsid that contain capsid amino acid sequences. Some of these larger proteins appear as discrete polypeptides only slightly bigger than the capsid, and we noted previously that these also contain amino acid sequences of the capsid. Presumably these polypeptides (such as band III of Fig. 3) also contain some sequences of the viral envelope protein encoded by the cistron adjacent to that of the capsid. It has been shown that the capsid protein can be covalently linked to viral envelope polypeptides when cells are infected with a temperature-sensitive mutant of Sindbis virus and incubated at nonpermissive temperatures (20). We have no direct evidence from data presented here that the in vitro system is making discrete viral envelope proteins. However, both the size distribution of the polypeptides made in vitro and the results from the immunoprecipitin study suggest that viral envelope cistrons were translated in vitro. We are currently analyzing the tryptic peptide fingerprints of the larger-size discrete proteins formed in vitro and the two envelope proteins purified from virions in order to map the cistrons in the 26S RNA.

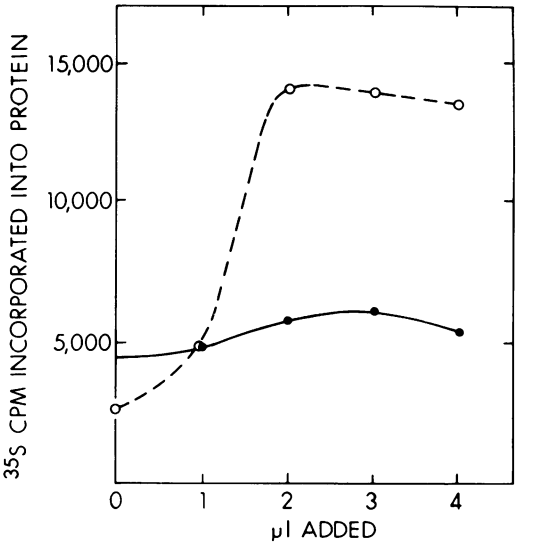

FIG. 9. *SDS-polyacrylamide gel pattern of proteins precipitated by anti-Sindbis virus antibodies. Samples were from precipitates of reactions described in Table 3. Samples 1 and 2, Prior to addition of antiserum from reaction mixtures containing no RNA and intracellular viral-specific RNA, respectively. Samples 3 and 4, Precipitates from reaction mixtures that had intracellular viral-specific RNA and were treated with anti-Sindbis serum and anti-TN9 serum, respectively. Refer to experimental procedures and Table 3 for other details.*

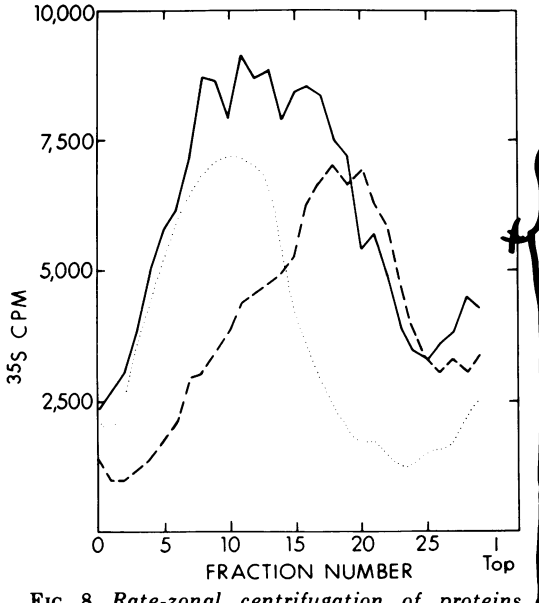

FIG. 8. *Rate-zonal centrifugation of proteins formed in vitro with low and high amounts of S-100 reticulocyte fraction. Symbols: ---, From mixture containing 5 μliters of S-100 and 8 μg of intracellular viral-specific RNA; —, from mixture containing 25 μliters of S-100 and 8 μg of intracellular viral-specific RNA. The total volume of reaction mixture was 50 μliters. See Fig. 4 for explanation of the dotted line and experimental details.*

The sequences of nucleotides in the 26S RNA are also present in the virion RNA, but the translation of virion RNA by our cell-free extracts mainly produced polypeptides that were larger and possibly distinct from those formed by 26S viral RNA. This result implies that the 26S region of the larger virion RNA is not readily accessible to the initiation factors of the in vitro system, possibly bacause the 26S sequences are near the 3′ terminal portion of the virion RNA. Both virion RNA and 26S RNA contain poly(A) sequences (9, 10) that presumably are at or near the 3′ terminal regions of the messenger RNAs (8). The proteins formed by virion RNA in the cell-free system were not recognized by antibodies directed against the virion, and we suspect that the large polypeptides translated from virion RNA are the early proteins made when Sindbis virus infects cells—for example, the RNA replicase. To confirm this, we are attempting to isolate replicase from infected cells in sufficient quantity to make a tryptic fingerprint.

We were surprised by the efficiency of the in vitro system in producing so much authentic

TABLE 3. *Immuno precipitation of viral proteins formed in vitro by ascites extracts*

Prepn of RNA used in reaction mixture	Antiserum used	Protein added (counts/min)	Protein in precipitin (counts/min)	Percent in immuno-precipitin
Endogenous (none added)	anti-Sindbis	5,000	46	0.9
	anti-TN9[a]	3,333	35	1.0
Virion RNA	anti-Sindbis	8,125	134	1.6
	anti-TN9	5,417	79	1.3
Intracellular viral-specific RNA	anti-Sindbis	15,571	2,050	13.3
	anti TN9	10,314	888	8.6

[a] Sera from rabbits given trinitrophenyl bovine gamma globulin (a gift of J. R. Little).

capsid. In vivo, the capsid is presumed to form by a proteolytic cleavage of a polypeptide chain that has been initiated only at the 5′ terminus of the polycistronic mRNA. We were reluctant to conclude that proteolysis was occuring so well in vitro because (i) the ascites cell-free system fails to show a proteolysis of the polypeptides translated from encephalomyocarditis virus and poliovirus RNAs even though viral proteins of these viruses are formed by a protease activity, and (ii) capsid was produced with Sindbis virus RNA in vitro in the presence of two known inhibitors, phenylmethane sulfonyl fluoride and L-1-tosylamide-2-phenylethyl chloromethyl ketone, of proteases.

We would conclude from the studies presented here that in vitro formation of capsid protein depends upon (i) the nature of the RNA, (ii) the source of the cell-free system, and (iii) the components of the cell-free system. Thus, even though all three kinds of viral RNAs used here contain the capsid cistron, the relative amount of capsid produced is quite varied. This can result either from a difference in the site of initiation on the different RNAs or a difference in the secondary structure that somehow allows for translation to selectively terminate at the end of the capsid cistron. As suggested above, we propose that the cistron for capsid is in an internal position in the 42*S* virion RNA but is at the 5′ end of the 26*S* RNA. This difference could account for the expression of capsid cistrons in the two RNAs. The nature of the "33*S*" RNA is an enigma, but there is some suggestion that it is the 26*S* species in an altered conformation (J. H. Strauss, personal communication). The profile of the in vitro polypeptides made by "33*S*" RNA that was analyzed on the urea-sucrose gradient suggests either that the "33*S*" species contains more genetic information than 26*S* or the conformation of the "33*S*" species allows for more read-through of the putative termination region and less release of discrete capsid.

As noted in Fig. 3, the CFSII system from rabbit reticulocytes makes many more proteins than does the ascites system, and these proteins are larger in molecular weight. A likely explanation for this difference is that there are more of the essential components required for in vitro protein synthesis in the CFS II system than in the ascites extracts. In fact, increasing the efficiency of the CFS II extracts by adding more S-100 extract led to increased amounts of protein as well as larger polypeptides. One interpretation of these data is that the discrete proteins we observed are the result of some limiting factor(s) in the in vitro system. For example, a limiting amount of a tRNA species would slow down translation and could lead to discrete terminations. Addition of extra tRNA from rat liver was without effect in our system, but the limiting tRNA may be unique to the reticulocyte cell. A slower rate of translation is also believed responsible for premature termination of the in vitro translation of encephalomyocarditis virus mRNA (13). One of the mechanisms proposed for these early terminations is an endonucleolytic digestion of the mRNA under conditions where there is slower movements of ribosomes along the RNA. We postulated a similar model in trying to account for discrete proteins of Sindbis in the in vitro reaction, suggesting that the added mRNA was either fragmented or became fragmented in the in vitro reaction (5).

However, neither the "limit tRNA" model nor a fragmented mRNA is totally satisfactory because we have found that intracellular viral-specific RNA from a temperature-sensitive mutant does not make a discrete capsid protein in vitro at 39 C even though capsid appears at 29 C (6). It is difficult to reconcile this result with either of the models. Another problem with these models is that they predict some discrete shorter polypeptides when virion RNA is translated, and these did not appear in significant amounts.

We are left with the conclusion that there is something quite distinctive about the translation of the capsid protein cistron. In the follow-

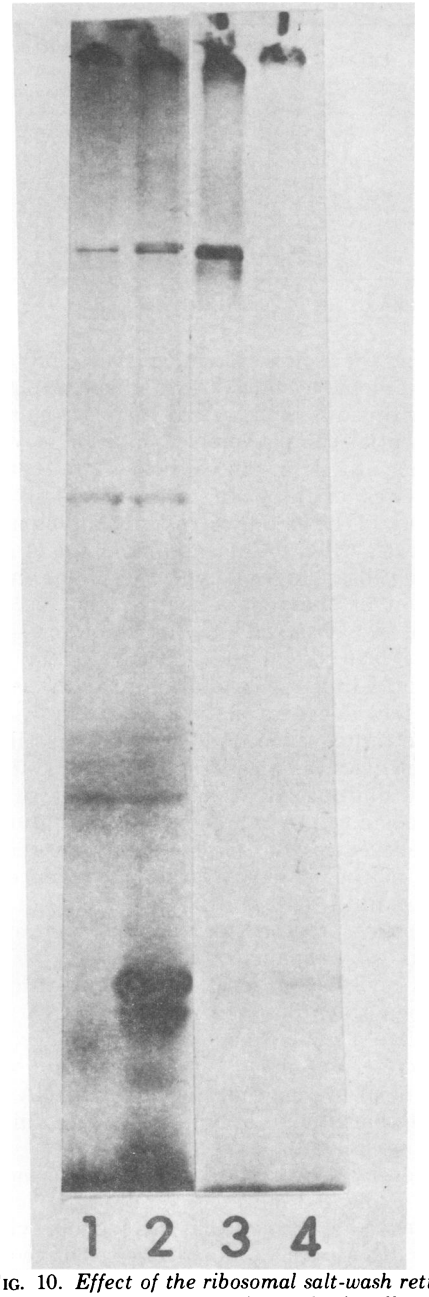

FIG. 10. *Effect of the ribosomal salt-wash reticulocyte fraction on in vitro protein synthesis. All reaction mixtures contained a total volume of 0.025 ml and were identical in composition except for the amount of salt-wash fraction (see ref. 5 for details). Symbols:* ●, *With 1.2 μg of intracellular viral-specific RNA added;* ○, *with no added viral RNA.*

ing paper (15) we discuss several alternatives for capsid formation based on in vitro translation of RNA from a Sindbis virus temperature-sensitive mutant.

ACKNOWLEDGMENTS

This research was supported by a Public Health Service grant (CA-14311-01) from the National Cancer Institute and GB38657 from the National Science Foundation.

We thank Irving Boime for generously providing us with the cell-free extracts from Krebs ascites cells and for his advice and suggestions in many aspects of this work.

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