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Protein Synthesis Directed by an Arbovirus

ROBERT M. FRIEDMAN

Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

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In contrast to chick embryo fibroblast protein synthesis, the bulk of the protein synthesis directed by Semliki Forest virus is carried out on membranes. Under conditions where more than 95% of cell protein synthesis was inhibited, viral polysomes could be demonstrated. Viral protein appeared to be produced on polysomes associated with nascent ribonucleic acid strands still attached to the base-paired, double-stranded replicative form of the virus. Very rapid incorporation of virus protein into 140*S* virus core particles was also demonstrated.

Although the synthesis of ribonucleic acid (RNA) by the group A arbovirus, Semliki Forest virus (6, 17, 18), as well as the elaboration of intracellular viral forms (5), and the formation of the virion (1, 5), have been extensively studied, few reports have been published about protein synthesis by any other member of this group (11). Studies of protein synthesis by these organisms is also of considerable interest, since these studies are necessary to provide a better understanding of the replication process.

In this investigation, marked differences between Semliki Forest virus (SFV) protein synthesis and protein synthesis of the host cell, chick embryo fibroblasts (CEF), were found. Although the bulk of CEF protein synthesis appears to be carried out on polysomes which are not closely bound to membranes (20), much viral protein production is carried out on membrane-associated polysomes. Under conditions where more than 95% of the cell protein synthesis was inhibited, viral polysomes could be demonstrated. The double-stranded replicative form of the virus was present in the virus polysome fractions. Very rapid incorporation of virus protein into intracellular 140*S* virus core particles was also noted.

MATERIALS AND METHODS

Virus pools, chick cell cultures, and sucrose density gradient analysis. The methods used for preparation of virus pools, chick cell cultures, and sucrose density gradient analysis, as well as the analytic procedures employed, have been described in detail elsewhere (5, 6). Protein was assayed by the method of Lowry et al. (10).

Infection of cells. CEF were treated with 2 μg/ml of actinomycin D for 2 hr at 37 C. After washing twice, the cells were incubated overnight at 4 C with SFV at a virus-cell multiplicity of 80:1. Infection was initiated by warming to 37 C (6).

Reagents. Partially purified chick interferon was prepared by the Fantes method (4). The interferon used in these studies was donated by Dr. Fantes, Glaxo Ltd., Stoke Poges, Bucks, England. The pool used contained 180 μg of protein/ml and 10,000 units of interferon per ml (1 unit of interferon inhibited SFV plaque development by 50%). Actinomycin D was donated by Merck, Sharp, and Dohme Research Laboratories (Div. of Merck & Co., Inc., Rahway, N.J.). Uridine-5-^{3}H (20 c/mmole), and ^{14}C and ^{3}H reconstituted protein hydrolysates were purchased from Schwarz Bio Research (Orangeburg, N.Y.).

Polysome preparation. CEF were washed five times with saline and removed from 100-mm plastic culture dishes by scraping into reticulocyte buffer [(RSB) 0.01 M KCl, 1.5×10^{-3} M $MgSO_4$, and 0.01 M tris(hydroxymethyl)aminomethane (Tris), *p*H 7.4]. The cells were broken with three strokes of a Dounce homogenizer or by treatment with 0.5% Nonidet P40 (NP40) for 10 min (16). The homogenate was sedimented for 10 min at 800 × *g*, and the supernatant fluid was layered over a 15 to 30% sucrose gradient in RSB and sedimented as indicated in the text. Fractions were collected and were assayed for optical density (OD) at 280 mμ and for acid-precipitable radioactivity in a Packard Tri-Carb liquid scintillation spectrometer (5).

RNA extraction. Sucrose density gradient fractions were pooled and extracted once with 1% sodium dodecyl sulfate (SDS), at 37 C for 2 min, and twice with phenol at 60 C. The extracted RNA was precipitated with 2 volumes of alcohol, and, after 16 hr at −20 C, the precipitate was collected by sedimentation. The RNA was resuspended in a mixture of 0.1 M KCl, 0.01 M Tris (*p*H 7.1), and 0.001 M ethylenediaminetetraacetic acid (EDTA), and then was analyzed on a 6 to 30% sucrose gradient.

RESULTS

Polysomes of uninfected chick embryo fibroblasts (CEF). CEF cultures, prepared 18 hr prior to use, were pulse-labeled for 3 min with reconstituted ^{14}C protein hydrolysate. Cytoplasmic fractions were prepared without de-

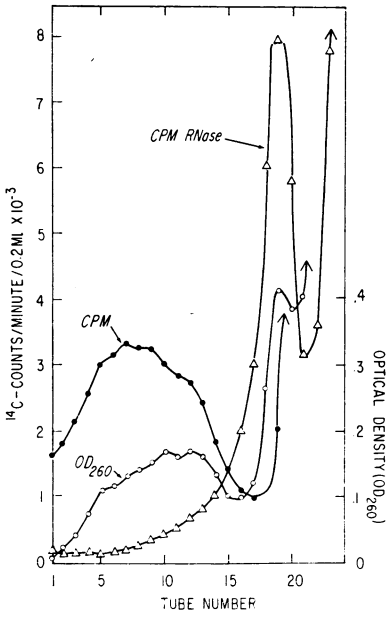

FIG. 1. *Chick cells (5×10^7) were incubated for 3 min in amino acid-free medium with 50 μc/ml of ^{14}C reconstituted protein hydrolysate. The cells were washed, scraped into RSB (0.01 M KCl, 1.5×10^{-3} M $MgSO_4$, 0.01 M Tris, pH 7.4), and disrupted with a Dounce homogenizer. After sedimentation at 800 × g for 10 min, 0.2 ml of the cytoplasmic extract was layered over a 15 to 30% sucrose gradient prepared in RSB and sedimented at 38,000 rev/min for 1 hr in an SW39 rotor. Fractions (0.2 ml) were collected by puncturing the bottom of the tube. The collected fractions were analyzed for OD at 260 mμ (OD_{260}) or for acid-precipitable radioactivity. An 0.2-ml portion of the extract was treated with ribonuclease (1 μg/ml at 0 C for 10 min) before sedimentation. The OD peak for chick ribosomes was assigned a value of 74S. The OD curve of the ribonuclease-treated extract closely followed the pattern seen for the radioactivity of this fraction. The bottom of the gradient is to the left. The pellet was suspended in buffer and found to contain a total of 9,182 acid-precipitable counts/min before ribonuclease treatment.*

tergents and were sedimented in a 15 to 30% sucrose gradient. The collected gradient fractions were analyzed, and the results (Fig. 1) indicated that less than 15% of the total acid - precipitable radioactivity in the gradient, excluding the soluble protein, was present in the pellet before ribonuclease treatment. The bulk of the radiolabel and a broad peak of OD were present in the polysome region of the gradient (~200S), but they were transferred, before sedimentation in the sucrose gradient, to the 74S monosome region by treatment with 1 μg/ml of ribonuclease at 1 C (Fig. 1).

TABLE 1. *Effect of virus infection, actinomycin D, or interferon on protein synthesis in chick fibroblasts*

Treatment[a]	Specific activity (counts per min per μg of protein)	Per cent control specific activity
None	169	100
Interferon only	165	98
Virus only	18.9	11
Actinomycin D only	27.5	16
Virus, actinomycin D	15.3	9

[a] Chick cells (2.5×10^7) were treated with 1,000 units/ml of chick interferon for 8 hr at 37 C or with 2 μg/ml of actinomycin D for 2 hr. Cells were washed with Eagle's medium and infected with 2×10^9 PFU of Semliki Forest virus at 4 C for 16 hr. The cells were then placed at 37 C for 4 hr, washed with amino acid-free medium, and incubated for an additional 10 min, with 10 μc/ml of ^3H reconstituted protein hydrolysate in the same medium. Cells were washed and scraped from the monolayer into 2.5% perchloric acid. The precipitates were collected, acid washed again, dissolved in 0.3 N NaOH, and then analyzed for protein and radioactivity.

TABLE 2. *Effect of interferon treatment on protein synthesis in uninfected and virus-infected cells*

Treatment[a]	Specific activity (counts per min per μg of protein)	Per cent control specific activity
None	92.2	100
Actinomycin D only	15.4	17
Interferon, actinomycin D	14.1	15
Actinomycin D, virus	6.1	6.6
Interferon, actinomycin D, virus	1.4	1.5

[a] Chick cells were treated as described in Table 1. In the case of multiple treatments, the additions were in the order listed.

Effect of actinomycin D and interferon on protein synthesis in uninfected and infected CEF. Experiments were performed to estimate what portion of the protein synthesis in virus-infected cells was virus-directed 4 hr after infection. CEF monolayers were treated with 1,000 units/ml of interferon for 8 hr. Some of these monolayers were then treated with 2 μg of actinomycin D for an additional 2 hr. Treatment of CEF with actinomycin D, several hours after interferon addition, had no effect on the development of antiviral activity stimulated by interferon (19). After

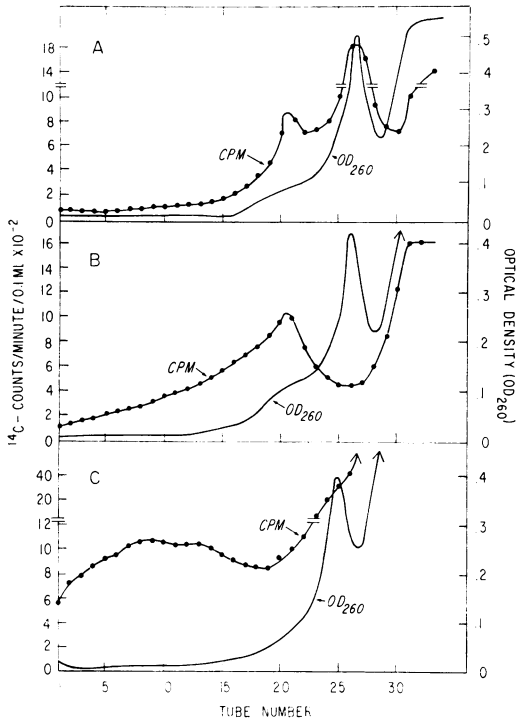

FIG. 2. *Cells (2.5 × 10^8) were treated with 2 μg/ml of actinomycin D for 2 hr at 37 C. One portion of the cells was infected with virus (see Materials and Methods). After 4 hr of incubation at 37 C, the cells were incubated for 3 min with 100 μc/ml of ^{14}C reconstituted protein hydrolysate. Cells were treated as previously described (Fig. 1). Extracts were sedimented at 25,000 rev/min, for 90 min, in an SW-25 rotor. Fractions (1.0 ml) were collected and analyzed as previously described. A. Infected actinomycin D-treated cells. The cells were treated with 1 μg/ml of ribonuclease, for 10 min at 0 C, before sedimentation. B. Infected actinomycin D-treated cells. The pellet contained 96,054 counts/min per 0.1 ml. C. Uninfected actinomycin D (2 μg/ml)-treated cells.*

actinomycin D treatment, some of the cells were infected as described in Materials and Methods, and, 4 hr after warming to 37 C, the cells and appropriate controls (Tables 1 and 2) were pulse-labeled with reconstituted ^3H protein hydrolysate.

The specific activity of the protein in each sample is listed in Tables 1 and 2. Treatment of cells with interferon had no effect on cell protein synthesis (9), but treatment with actinomycin D and with virus inhibited protein synthesis by 16 and 11% of the control, respectively (Table 1). The gradient patterns of uninfected actinomycin D-treated cells showed a marked decrease in polysome function and a high level of free monosomes (Fig. 2C). Treatment with both actinomycin D and virus did not significantly lower the level of protein synthesis when compared to treatment with virus alone (Table 1). Since the addition of actinomycin D to virus-infected cells would have significantly depressed any cell protein synthesis present, this observation suggested that most of the protein synthetic activity observed was due to virus. These data indicate that cell protein synthesis is strongly inhibited by virus infection, as well as by actinomycin D treatment, and that protein synthesis occurring 4 hr after SFV infection is largely viral.

Further studies with interferon (Table 2) support these findings. Infected CEF, pretreated with interferon and later with actinomycin D, had about one-quarter of the specific activity of virus-infected and actinomycin D-treated cells which had not received interferon treatment. Since interferon inhibited only virus protein synthesis (Table 1), the results indicated that 4 hr after infection, most of the protein produced was viral. The inhibition of protein synthesis (77%) by interferon treatment provides a minimal estimate of virus protein synthesis, as interferon only partially inhibits virus synthetic activities (13).

Protein synthesis in SFV-infected cells. Cytoplasmic extracts were prepared from SFV-infected cells in the same manner as was described for uninfected CEF (Fig. 1). Marked differences were noted in the gradient patterns obtained from infected cells as compared to the gradient patterns obtained for uninfected cells. In contrast to the pattern of uninfected cells (Fig. 1), more radioactivity (about 50%) was present in the pellet in the gradients from infected cells (Fig. 2B). A single peak of radioactivity was present at 140*S*; and, in addition, some radioactivity was present at 200 to 300*S*, presumably on polysome structures. Ribonuclease treatment before sedimentation (Fig. 2A) brought about two changes in this pattern. The radioactivity present at 200 to 300*S* in the untreated sample was no longer seen, and a new peak was present in the 74*S* monosome area. However, a peak remained at 140*S*, and this peak corresponded to the virus core particle, which contained only the 42*S* RNA of the mature virus. This 140*S* fraction was previously observed in, and purified from, the cytoplasm of SFV infected cells (5). A similar structure was seen in purified virus preparations treated with the proteolytic enzyme caseinase C (14).

Infected CEF were pulse-labeled with ^3H protein hydrolysate, the cytoplasm was treated with 0.5% NP 40, and the cytoplasmic fraction was

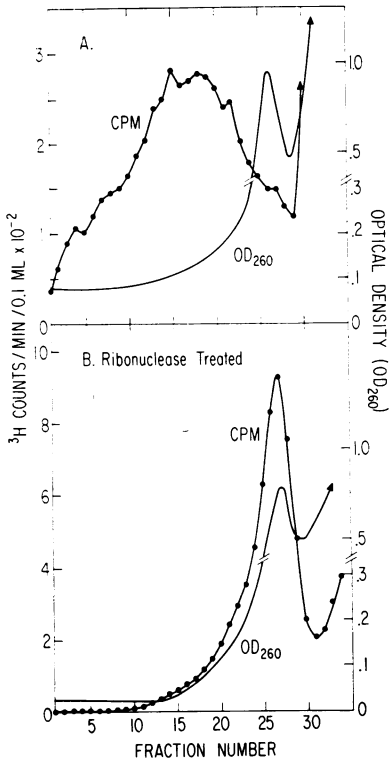

FIG. 3. *Infected cells 2.5 × 10⁸ were treated and radiolabeled as previously described (Fig. 1 and 2). The cells were extracted with 0.5% nonidet P40 and, after sedimentation at 800 × g for 10 min, were sedimented in an SW-25 rotor for 90 min at 25,000 rev/min. Fractions were collected and analyzed as previously described. A. Detergent-extracted cells. Pellet contained 872 counts/min per 0.1 ml. B. Extract treated with 1 μg/ml of ribonuclease, for 10 min at 0 C, before sedimentation.*

analyzed on sucrose density gradients. About 15% of the counts in the gradient were in the pellet (Fig. 3A). A broad 200 to 300*S* peak of radioactivity was observed and this gave rise to a 74*S* peak after ribonuclease treatment (Fig. 3B). As compared to the cytoplasm of cells fractionated by Dounce homogenization, the cytoplasm of the detergent NP40-treated cells lacked a prominent 140*S* peak. Detergent treatment had, therefore, two effects as compared to fractionation with the homogenizer (Fig. 1). The radioactivity previously present in the pellet was released and now appeared in the gradient fractions as a 200 to 300*S* peak, whereas the 140*S* peak was no longer prominent. The 140*S* virus core previously was shown to be sensitive to detergent treatment (5).

In contrast to protein synthesis in uninfected cells (Fig. 1), protein synthesis in SFV infected CEF took place, to a great extent, on membranes (Fig. 2B). Without detergent treatment, the radioactivity associated with the viral polysomes appeared mostly in the pellet. After detergent treatment, a typical polysome pattern was obtained in extracts of infected CEF (Fig. 3A). Since more than 75% of the protein synthesis in the actinomycin D-treated cells 4 hr after infection was inhibited by interferon treatment (Table 2), most of this activity in the 200 to 300*S* polysome fraction was probably virus-directed.

Polysome-associated virus RNA. SFV infected cells were incubated for 20 min with ^{3}H-uridine, 2 and 4 hr after infection. A 20-min period was chosen because shorter pulses did not yield sufficient incorporation of isotope. Only the results from samples radiolabeled 2 hr after infection are shown, but those from samples radiolabeled 4 hr after infection were similar in all respects. Sucrose density gradient analysis of the ^{3}H-uridine labeled and NP 40 extracted cytoplasm yielded the pattern shown in Fig. 4A. The most prominent peak sedimented somewhat faster than the 74*S* ribosomes seen in the OD pattern. Some radioactivity was present in the 200 to 300*S* region of the gradient, the fractions previously shown to contain the virus polysomes. The peak of radioactivity sedimenting at about 80*S* corresponded to a cytoplasmic fraction previously shown to contain only 26*S* viral RNA (5).

When the cytoplasmic extract was treated with ribonuclease before its sedimentation (Fig. 4B), the radioactivity previously seen in the 200 to 300*S* region was no longer present. The radioactivity in the 80*S* region was diminished, but most of the radiolabel previously present in the 80*S* peak had shifted to the peak seen at about 60*S*. Such behavior was described previously in detail (5). In cells extracted and sedimented in magnesium-free medium containing 0.01 M EDTA, both the 200 to 300*S* and the slower sedimenting peaks were absent (Fig. 4C). The sensitivity of the 200 to 300*S* peak to treatment with ribonuclease (Fig. 4B) or EDTA (Fig. 4C) and the fact that this region of the gradient was shown to contain polysomes (Fig. 3A) suggested that the ^{3}H-uridine labeled viral RNA seen in the 200 to 300*S* region was associated with ribosomes, probably as viral RNA serving a messenger function.

The viral RNA from the 200 to 300*S* region of the gradient (fractions 8–14, Fig. 3A) was extracted with phenol and SDS, alcohol precipitated, and resuspended in buffer. It was then layered with marker chick ribosomal RNA, over a 6 to 30% sucrose gradient, and sedi-

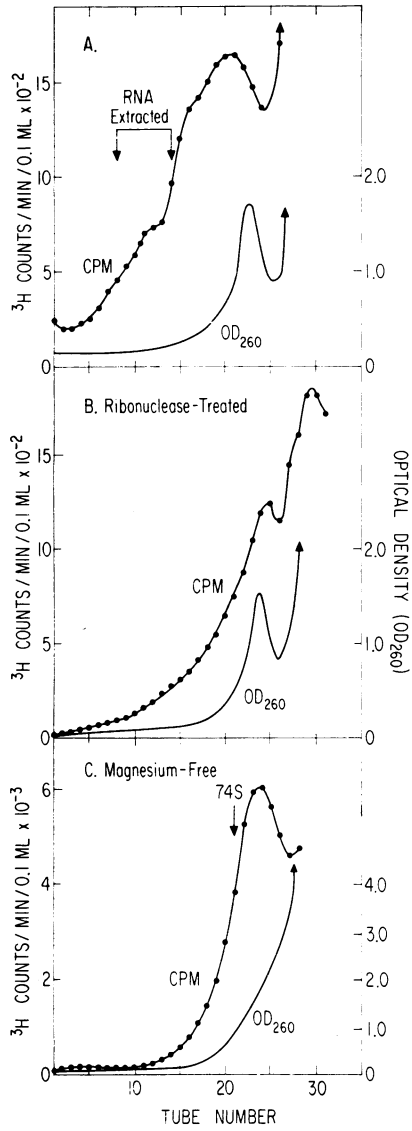

FIG. 4. *Cells were actinomycin D-treated and infected as described previously. After 2 hr, the cells were pulse-labeled for 20 min with 100 μc of uridine-5-³H. Cells were extracted with nonidet P40 in RSB (A and B), or in 0.01 M KCl, 0.01 M Tris, pH 7.4, and 0.001 M EDTA (C). Extracts were sedimented, and fractions were collected and analyzed as previously described. A. Extract of infected cells. RNA was extracted from fractions 8–14. B. As (A), except that cells were treated with 1.0 μg/ml of ribonuclease for 10 min at 0 C before sedimentation. C. Extract of cells in Mg++-free medium. No 74S ribosome peak is seen in the OD pattern. The 74S peak from a simultaneously run extract in RSB is shown.*

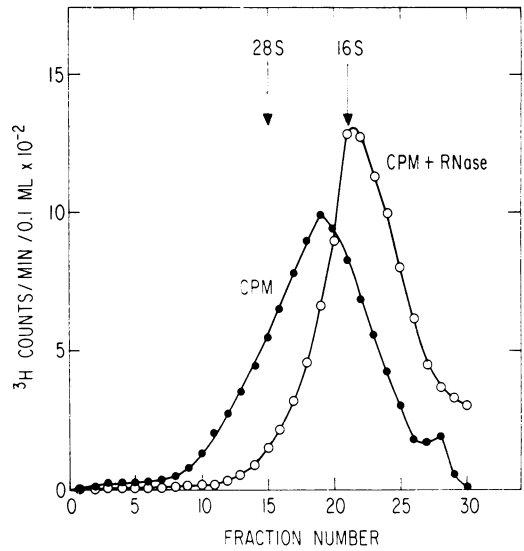

FIG. 5. *RNA was extracted from fractions 8 to 14 (Fig. 4) by the phenol-SDS method and was alcohol-precipitated. An 0.2-ml sample was mixed with carrier chick ribosomal RNA, layered over a 6 to 30% sucrose gradient in a mixture of 0.1 M KCl, 0.01 M Tris (pH 7.1), and 0.001 M EDTA, and sedimented at 38,000 rev/min for 3 hr. Another 0.2-ml sample was treated with 2 μg/ml of ribonuclease for 30 min at 37 C before sedimentation. Fractions were collected and analyzed as previously described. The bottom of the gradient is to the left; 28S and 16S denote the OD peaks of carrier ribosomal RNA.*

mented. The sucrose gradient patterns obtained are shown in Fig. 5. A fairly broad peak of radioactivity was seen at about 20S. When treated with ribonuclease before sedimentation, the peak proved to be ribonuclease-resistant, but had become sharper and had shifted to 16S. This behavior corresponded to that previously shown for the replicative intermediary (RI) for SFV (R. M. Friedman, *unpublished data*).

DISCUSSION

The sucrose density gradient pattern observed for cytoplasmic extract of uninfected CEF was similar to that seen for some other cell systems (15, 20). About 15% of the protein synthesis appeared to be membrane-bound. Virus infection radically altered this pattern.

Studies employing actinomycin D and interferon in infected cells indicated that the bulk of the protein synthesis under these conditions was virus-directed. Much of this activity was carried out on membranes, in contrast to the pattern seen in uninfected cells. When the cytoplasm of infected cells was extracted with detergent, virus-specific polysomes were present in the 200 to

300S region of the gradient. When the viral RNA in these infected- and actinomycin D-treated cells was labeled with ^{3}H-uridine, and the viral RNA was extracted from the 200 to 300S fractions, the RI of the virus was found.

In previous studies, evidence was presented which supported the conclusion that the RI of SFV consisted of double-stranded RNA with nascent viral RNA (5). With prolonged sedimentation, the RI was polydisperse, and, with ribonuclease treatment of the phenol-SDS extracted RI, a base-paired ribonuclease-resistant form was produced (R. M. Friedman, *unpublished data*). Evidence presented in this study suggested that the RI was also associated with virus protein synthesis. The RI and virus RNA-dependent RNA polymerase are, like virus protein synthesis, membrane-associated (5, 12).

Since the viral polysome is ribonuclease-sensitive (Fig. 3B and 4B), the viral RNA serving a messenger function may be the ribonuclease-sensitive portion of the RI, the nascent viral RNA. This would suggest that the nascent RNA rapidly becomes ribosome-associated and assumes a messenger function even before its completion on the double-stranded template. The existence of such double-stranded viral RNA-polysome complexes may explain the previously observed close relationship between viral RNA and protein synthesis (8). An analogous deoxyribonucleic acid (DNA)-RNA-polysome complex has been demonstrated in a cell-free system with *Escherichia coli* extracts and T2 phage DNA (3). Very recently, double-stranded viral RNA was found in the polysome fractions of *E. coli* infected with bacteriophage R17 (7).

The rapid labeling of the 140S virus core structure with radioactive amino acids indicates that at least one virus structural protein is incorporated into elements of the virion almost as soon as protein is formed. This suggests that some remarkably efficient mechanism exists to carry out this association. At any rate, the 140S fraction (or virus core) was shown to contain only the 42S viral RNA species of viral RNA, the synthesis of which requires at least 10 min (5, 6). Since the 140S core was labeled with radioactive amino acids within 3 min, it appeared to be formed by combination of new protein with viral RNA from a 42S RNA pool. The opposite situation was seen in poliovirus infection, where new RNA was incorporated into the virion with protein that had been previously produced (2).

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