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Regulation of Protein Synthesis in HeLa Cells

III. Inhibition During Poliovirus Infection

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The mechanism of inhibition of host cell protein synthesis by poliovirus has been studied by examining the behavior of host polyribosomes and polyribosome-associated messenger ribonucleic acid (mRNA). Virus infection appears to result in a specific inhibition of the initiation of translation of host cell mRNA. The site of inhibition does not appear to be mRNA itself. Human cells respond to virus infection by producing a factor antagonistic to the virus inhibition which promotes the initiation of host mRNA translation. The production of the host factor is sensitive to actinomycin; however, the behavior of the host cell factor and that of host mRNA appear distinctly different.

A fundamental question in the study of eukaryotic macromolecular metabolism is the regulation of protein synthesis. Unlike bacteria, messenger ribonucleic acid (mRNA) is long lived in eukaryotic cells, and the regulation of translation appears to be an important aspect of the control of cell metabolism (5, 8, 12, 22). An interesting example of selective control of translation occurs during the infection of cells with picornaviruses. In this case, host protein synthesis is rapidly inhibited, whereas the components of the protein synthetic machinery remain capable of translating viral ribonucleic acid (RNA) (14). Previous work has also indicated that the inhibition of host protein synthesis is not simply due to nucleolytic activities which degrade host mRNA (24).

In contrast to rodent cells, host RNA synthesis in human cells continues for at least 2 hr after infection with picornavirus (6, 11). Host protein synthesis in human cells also continues for much longer than in infected rodent cells in which host RNA synthesis is rapidly inhibited (11). However, if host RNA synthesis is blocked in human cells with actinomycin, a much more rapid shut-off of host protein synthesis ensues (9, 14, 24). Apparently, continued host RNA synthesis permits the production of a factor which is antagonistic to the inhibitory function produced by the virus. In these experiments, the behavior of the host protein synthetic machinery was examined in the presence and absence of actinomycin to study both the mechanism of inhibition and the nature of the cellular response.

The experiments reported here were conducted in the presence of guanidine which suppresses poliovirus replication (15). The experimental results suggest that an inhibitory factor is produced by the virus which interferes with the entry of ribosomes into the polyribosomes. The cell response is apparently the production of a factor which promotes the association of ribosomes with polyribosomes.

MATERIALS AND METHODS

Cell growth and infection. HeLa S3 cells were grown in suspension culture in Eagle's medium with 7% horse serum (2). Poliovirus stocks were prepared as described by Willems and Penman (24). Infections were initiated by suspending the cells in virus stock [4×10^9 plaque-forming units (PFU)/ml] at a concentration of 4×10^7 cells/ml in the presence of 3 mM guanidine. After incubation at room temperature for 15 min, the cell suspension was diluted to 2×10^6 cells/ml with warm medium containing 3 mM guanidine. The timing of the infection was begun at this point. When the cells were treated with actinomycin D, the drug was present during the virus adsorption step.

Cell fractionation. Cells were fractionated as previously described (13). Cells were washed once with Earle's saline (3), swollen for 10 min in hypotonic buffer RSB [0.01 M NaCl, 0.0015 M $MgCl_2$, 0.01 M tris(hydroxymethyl)aminomethane(Tris), *p*H 7.4], and disrupted with 10 strokes of a tight-fitting stainless-steel Dounce homogenizer. Nuclei were removed by centrifugation at 2,000 rev/min for 2 min.

The polyribose content of cytoplasmic extracts was analyzed by sucrose density gradient centrifugation as previously described (14) by using either low ionic strength (RSB) or high ionic strength buffer (HSB, 0.5 M NaCl, 0.05 M $MgCl_2$, 0.01 M Tris, *p*H 7.4). In experiments in which polysomes were to be analyzed on sucrose gradients of high ionic strength, 0.25 volumes of fivefold concentrated, high-salt buffer HSB were added to the cytoplasmic extracts prior to

centrifugation. The resulting optical density profiles were recorded with a continuous-flow ultraviolet monitor.

Polyribosome RNA was prepared by pooling the appropriate fractions of sucrose-RSB gradients and adding ethylenediaminetetraacetic acid (EDTA) to 0.01 M and sodium docecyl sulfate (SDS) to 0.5%, followed by 2 volumes of ethanol. The resulting precipitate was dissolved in SDS buffer (0.1 M NaCl, 0.001 M EDTA, 0.5% SDS, 0.01 M Tris, *p*H 7.4) and centrifuged through 15 to 30% sucrose gradients in SDS buffer. No change in sedimentation profile of RNA was observed if the sample was deproteinized by phenol extraction prior to analysis in sucrose-SDS gradients.

Radioactive labeling. Uridine-*2-*^{14}C (50 mCi/mmole), uridine-*5-*^{3}H (20 Ci/mmole), leucine-*4,5-*^{3}H (15 Ci/mmole), and ^{14}C-amino acid mixture ("reconstituted algal protein hydrolysate," approximately 100 mCi/mmole) were purchased from Schwartz Bio-Research, Inc.

mRNA was selectively labeled by incubating cells at a density of 2×10^6/ml with actinomycin D at 0.04 μg/ml for 25 min prior to the addition of ^{3}H-uridine (5 μCi/ml). The labeling period before viral infection was 90 to 120 min.

Fractions from sucrose and CsCl gradients were assayed for acid-precipitable radioactivity as previously described (5).

Acrylamide gel electrophoresis of protein. Electrophoresis of proteins was carried out by a modification of the method of Shapiro et al. (20) as described by McCormick and Penman (12). Samples were precipitated with 10% trichloroacetic acid; the precipitate was suspended in electrophoresis buffer [0.04 M Tris, 0.02 M sodium acetate (*p*H 7.4), 0.002 M EDTA, 10% (v/v) glycerol, 0.5% (w/v) SDS, 0.5 M urea, 0.1% (v/v) mercaptoethanol] and dialyzed for 16 hr against the same buffer. Electrophoresis was carried out through 5-cm, 7% acrylamide gels at 7 v/cm for 3 hr. Gels were polymerized, fractionated, and prepared for scintillation counting as described by Weinberg et al. (23).

Actinomycin D was a gift of Merck, Sharpe and Dohme. Cycloheximide was purchased from Calbiochem.

RESULTS

Decay of host cell polyribosomes in number and size after infection. The decay of host cell polyribosomes during virus infection is shown in Fig. 1. Guanidine is used to prevent virus multiplication, and the poliovirus-induced inhibitory activity appears at a linear rate under these conditions (24).

The relatively slow decline in host protein synthesis (Fig. 1) is apparently due to a vigorous competition between virus-produced inhibitor and a cell-mediated response. Actinomycin added at 60 min results in a rapid decline in active ribosomes with a half-life of less than 30 min. Actinomycin added at 75 min results in an even more rapid inhibition of host cell protein synthesis.

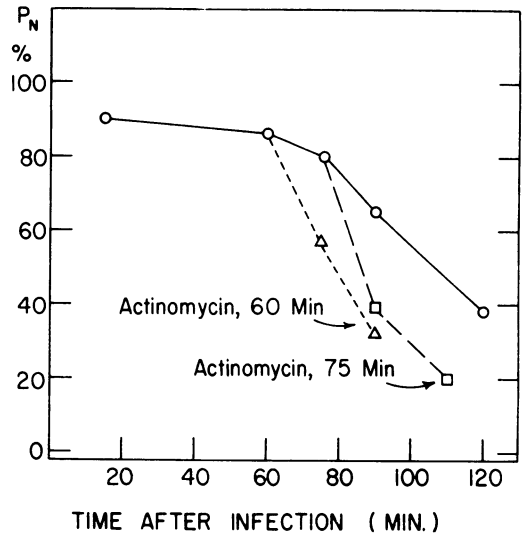

FIG. 1. *Kinetics of actinomycin-induced polysome decay in polio-infected cells. Cells (1.4×10^8) were infected with polio in the presence of guanidine and diluted to 70 ml. At 60 and 75 min after infection, respectively, 14-ml samples were removed and incubated with 5 μg of actinomycin per ml. At the times shown, 7-ml samples were removed from the various cultures and chilled. Cytoplasmic extracts were analyzed on RSB-sucrose gradients. Optical density (OD) at 260 nm was monitored during collection, and the fraction of total ribosomal OD contained in polysomes (P_N) was calculated as previously described (24).*

The experimental results shown in Fig. 2 and 3 indicate that the size of polyribosomes decreases after infection with poliovirus in the presence of actinomycin. Figure 2 shows the change in sedimentation profile of polyribosomes during decreasing host protein synthesis. The accumulation of single ribosomes after extensive inhibition tends to obscure the small polyribosomes. The distribution of monomeric ribosomes is obtained by adding ^{14}C monomeric ribosomes to the extract and using their distribution to obtain an accurate correction for the overlap between monomers and polyribosomes. Comparing the superposed, corrected distribution in Fig. 2 (right), it is apparent that even at the relatively early time of 65 min after infection, polyribosomes sediment more slowly than at 25 min after infection.

The distribution in sedimentation of structures actually active in protein synthesis was measured by pulse labeling with ^{3}H-leucine at various times after infection (Fig. 3). Each sucrose gradient was calibrated by adding polyribosome from uninfected cells which had been pulse labeled with

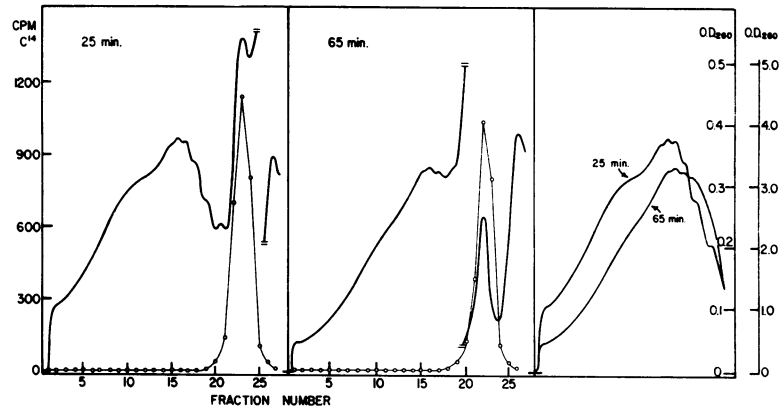

FIG. 2. *Sedimentation properties of polysomes in infected cells. Cells (4 × 10⁷) were infected in the presence of 5 μg of actinomycin per ml and 3 mM guanidine. Half the culture was chilled at 25 min and half at 65 min post-infection, respectively. Cytoplasmic extracts were prepared and mixed with samples of ^{14}C-labeled ribosome monomers. (The latter were prepared from cells grown for 24 hr in the presence of 2 × 10⁻⁶ M uridine-2-^{14}C by RSB-sucrose gradient fractionation of cytoplasmic extract.) The extracts and ribosome sedimentation marker were centrifuged through RSB-sucrose gradients in the SW25.3 rotor at 25,000 rev/min for 120 min at 5 C. The contribution of inactive monomers to polyribosome optical density was estimated as follows. The relative amount of radioactivity in each gradient fraction compared to the radioactivity in the monomer zone was calculated. The same proportion of the optical density (OD) of each fraction compared to the peak height of the monomer OD was taken to be the contribution of the monomers to polysome OD. The corrected absorbance profiles (monomer contribution subtracted) are shown, superposed, in the right hand figure.*

^{14}C amino acids to serve as a sedimentation standard. The distribution of ^{14}C-labeled polyribosomes from uninfected cells is completely superimposable between the different gradients, indicating that the ^{3}H distribution from infected cells may be directly compared.

The control shows polyribosomes from mock-infected cells which exhibit no change in sedimentation distribution. In contrast, the average sedimentation rate of active structures in infected cells has decreased significantly by 90 min. It should be noted that even relatively small changes in sedimentation velocity can represent large changes in the number of ribosomes associated with an mRNA molecule.

The data in Fig. 3 also indicate that the translation rate of ribosomes on mRNA in infected cells is approximately the same as in control cells since the ratio of radioactivity in nascent polypeptide chains on polyribosomes to that in released protein at the top of the gradient is similar. This is in agreement with a previous report (21).

mRNA on host cell polyribosomes. The decreased rate of sedimentation of host cell polyribosomes in virus-infected cells suggests that the ribosome packing on mRNA has been decreased. The following experiment directly measures the ratio of mRNA to ribosomes in polyribosomes. Cells were labeled prior to infection in the presence of a low level of actinomycin which suppresses the synthesis of ribosomal RNA (18, 19). Most of the radioactive material sedimenting with polyribosomes is mRNA, although a correction is made for labeled material which sediments in the polyribosome region but which is associated with EDTA-insensitive structures (16). After selective labeling with ^{3}H-uridine, the cells were infected in the presence of a high level of actinomycin.

The results in Fig. 4 show the sedimentation of labeled, heterogeneous mRNA obtained from polyribosomes. The amount of both mRNA and ribosomal RNA in polyribosomes decreases after infection, but the ribosomal RNA decreases much faster. The inset shows that the amount of ribosomal RNA per mRNA molecule declines to about one-half that found in control cells.

The criterion used for polyribosome-associated mRNA is the sensitivity of this material to EDTA added prior to sedimentation analysis of cytoplasmic extract. Thus all mRNA disappears from the polyribosome region of sucrose gradients after the addition of the chelating agent. A flat heterogeneous distribtution of RNA which is not mRNA remains in the polyribosome region and its contribution is subtracted (16). The identification of the EDTA-sensitive RNA as messenger was verified by using the criterion of isopycnic banding of glutaraldehyde-fixed polyribosomes in CsCl (1, 17). The size distribution of mRNA does not change noticeably during the time that polyribosomes decrease in size. Thus, the reduced

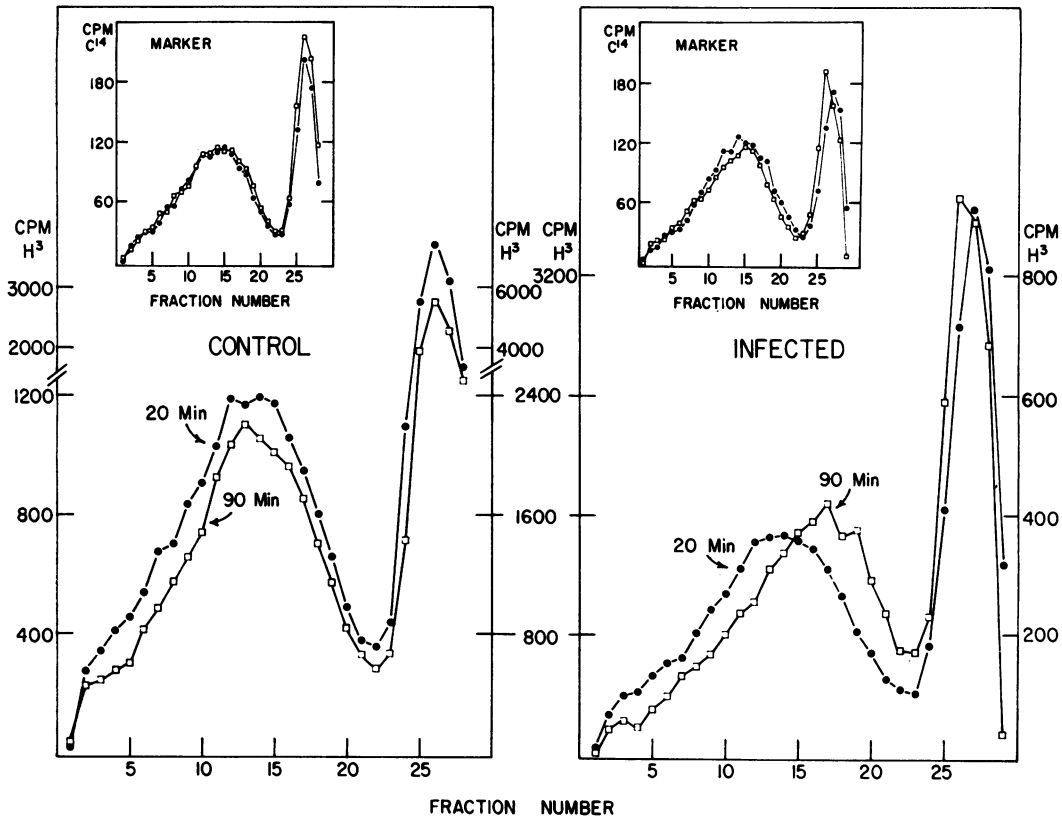

FIG. 3. *Sedimentation distribution of polysomes pulse-labeled with ^{3}H-leucine. Cells (4 × 10^7) were infected with poliovirus and diluted to a final volume of 20 ml with Eagle's medium with one-fourth normal concentrations of amino acids. Actinomycin, 5 μg/ml, and 3 mM guanidine were present throughout. A mock-infected control was treated identically, except that virus was omitted. At 20 min after infection, 7-ml samples of each culture were removed and incubated with 60 μCi of ^{3}H-leucine (15 mCi/μmole). After 60 sec, the cultures were chilled by pouring onto crushed, frozen Earle's saline. Additional 7-ml samples were pulsed at 90 min after infection in the same manner, except that 75 and 200 μCi of ^{3}H-leucine were used for the uninfected and infected samples, respectively. Each sample was then mixed with an equal portion of a culture of uninfected cells (2 × 10^7 total) which had been pulsed for 75 sec in amino acid-free Eagle's medium with 2 μCi of a mixture of ^{14}C-labeled amino acids. Cytoplasmic extracts were prepared, treated with 0.5% deoxycholate, and analyzed on HSB-sucrose gradients as described in Materials and Methods. Centrifugation in the Spinco SW25.3 rotor was at 25,000 rev/min for 135 min at 5 C. Left scale on each figure (—●—); right scale on each figure (—□—).*

sedimentation velocity appears to be the result of reduced loading of ribosomes and not to a reduction in the size of polyribosome-associated mRNA.

Size of polypeptides synthesized during host cell inhibition. The possibility that ribosomes prematurely detach from mRNA is next ruled out. Premature detachment during translation would result in a marked decrease in the size of newly synthesized polypeptides. Results in Fig. 5 show that the distribution in molecular weight of protein synthesized during infection is the same as that in uninfected cells. Protein labeled with ^{14}C-leucine from uninfected cells is mixed with protein labeled with ^{3}H-leucine in infected cells after substantial inhibition of protein synthesis has occurred. The proteins are analyzed together by electrophoresis in acrylamide gels in the presence of SDS and 0.5 M urea. Under these conditions, the electrophoretic mobility is determined almost entirely by the chain length of the polypeptide. No significant difference is observed in the distribution of radioactivity from infected and uninfected cells. In contrast, proteins obtained from cells where premature polypeptide termination has been produced by puromycin show a very different distribution in radioactivity with a much smaller average chain size.

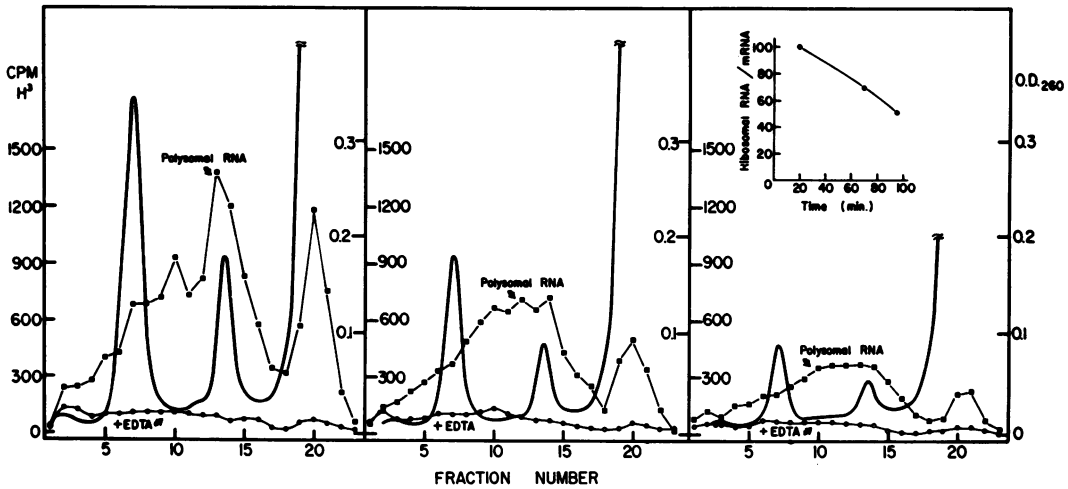

FIG. 4. *Relative amounts of ribosomes and mRNA in polysomes. Cells (7.2 × 10⁷) were labeled with 150 μCi ³H-uridine for 2 hr in the presence of 0.04 μg actinomycin per ml. The culture was infected with polio in the presence of 5 μg of actinomycin per ml and 3 mM guanidine. Samples were chilled at 20, 70, and 95 min after infection, and cytoplasmic extracts were prepared in RSB. Each extract was divided in equal parts and EDTA was added to half a final concentration of 10 mM. The six samples were analyzed on RSB-sucrose gradients (12 ml) centrifuged in the SW40 rotor at 40,000 rev/min for 45 min at 5 C. Optical density (OD) at 260 nm was monitored and fractions corresponding to structures sedimenting with the velocity of polysomes were pooled from each gradient. RNA was prepared from each sample (see Materials and Methods), centrifuged through SDS-sucrose gradients in the SW25.3 rotor for 17 hr at 25 C. OD profiles were determined and the fractions were analyzed for acid-precipitable radioactivity. OD in the ribosomal 28S from the polysomal RNA samples was determined with a planimeter. mRNA was defined as the radioactivity in polysome RNA fractions 1 to 18 after subtracting the radioactivity in those fractions from the corresponding EDTA-treated samples. Left panel, 20 min.; middle panel, 70 min.; right panel, 95 min.*

The sensitivity of this method of determining average polypeptide size is calibrated in the electropherogram shown in the bottom panel of Fig. 5. Polio-specific proteins derived from productively infected cells late in infection were analyzed together with ³H-labeled proteins from uninfected HeLa cells. The molecular weight of the poliovirus proteins are indicated. The calibration indicates that the observed effect of puromycin shown in the middle panel corresponds to a reduction of the average polypeptide length to about one-third to one-half of normal size.

If the reduction in ribosome loading on mRNA in virus-infected cells were due to premature release of ribosomes, a twofold reduction in the size of the polypeptide synthesized would be produced and would be detected by this method.

Rebuilding of polyribosomes by reducing translation rate. The following experiment shows that the inactive ribosomes present in the partially inhibited, infected cells can be mobilized onto polyribosomes. Previously it had been shown that low levels of cycloheximide can be used to reduce the rate of translation of ribosomes on mRNA and increase the loading of mRNA with ribosomes where the initiation of mRNA translation is rate limiting (5, 7, 12). The results in Fig. 6 show that reducing the rate of translation in polio-infected cells with cycloheximide increases the number of ribosomes in polyribosomes. Since the virus-induced disaggregation of polyribosomes can be partly reversed by reducing the rate of translation, ribosomes do not appear irreversibly altered in their capacity to associate with host mRNA.

Rate of host mRNA formation after virus infection. The response of the host cell which maintains host protein synthesis and which is suppressed by actinomycin (Fig. 1) suggests that some species of RNA is produced and inactivated at an elevated rate after infection. This is illustrated by the rapid decay of protein synthesis upon the addition of actinomycin. The factor produced has a half-life of apparently less than 30 min by 60 min after infection. However, the following experiment shows that host mRNA is not rapidly turning over and is apparently not the factor produced in response to infection.

Previous results have shown that mRNA is not labeled at an elevated rate during infection

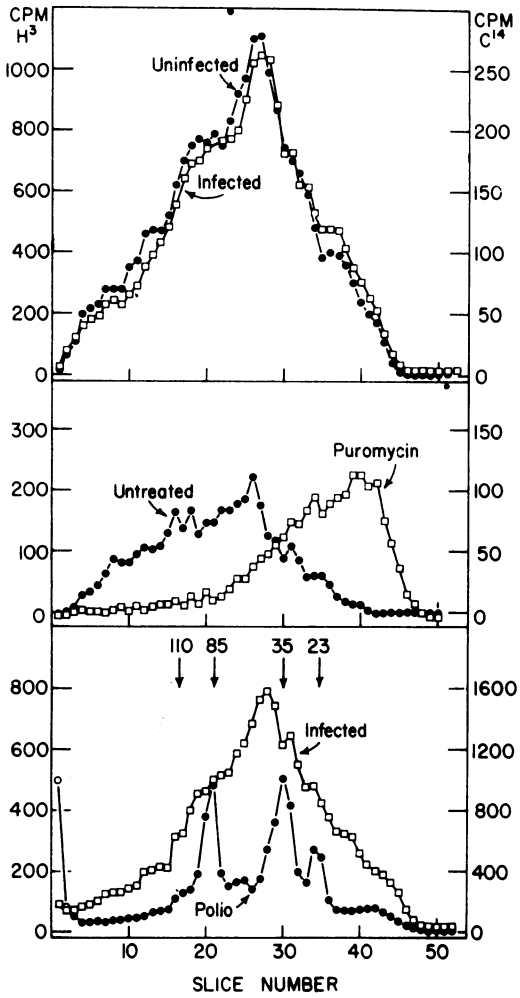

FIG. 5. *Size of proteins synthesized in uninfected, polio-infected, and puromycin-treated HeLa cells. Cells infected with polio (4 × 10⁷ in a final volume of 20 ml) in the presence of 5 μg of actinomycin per ml and 3 mM guanidine were labeled with 100 μCi of ³H-leucine from 70 to 130 min after infection; a mock-infected control was labeled in parallel with 5 μCi of ¹⁴C-leucine. A third culture (2 × 10⁷ cells) was treated for 10 min with 10 μg of puromycin per ml and then labeled for 50 min with 60 μCi of ³H-leucine. Cytoplasmic extracts were prepared from each, and portions of the infected sample were mixed with samples of the following: (top) mock-infected control; (middle) puromycin-treated culture; (bottom) ¹⁴C-labeled poliovirus-specific proteins, used as molecular-weight markers (a kind gift of M. Jacobson). Molecular weights are expressed as daltons × 10⁻³. The mixed extracts were then analyzed by gel electrophoresis as in Materials and Methods.*

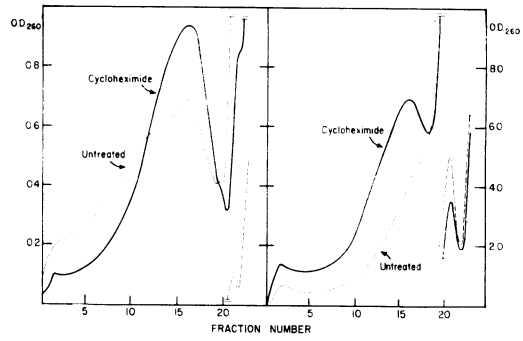

FIG. 6. *Partial reloading of polyribosomes in a low level of cycloheximide. Cells (6 × 10⁷) were infected in the presence of 5 μg actinomycin per ml and guanidine. Samples of 1.6 × 10⁷ cells were chilled at 15 and at 90 min after infection. Additional samples were removed at both times and incubated with 5 μg of cycloheximide per ml for 20 min and then chilled. Cytoplasmic extracts were prepared from each sample and analyzed on RSB-sucrose gradients as in Materials and Methods. Left, 15 min after infection; right, 90 min after infection. Untreated, broken line; cycloheximide 20 min, solid line.*

(24). However, unknown alterations in the pyrimidine pools might mask a significant stimulation in the rate of mRNA production. Nevertheless, the kinetics of labeling of mRNA during infection can serve to indicate whether these molecules have properties which are consistent with the metabolic behavior of the protein synthesis sustaining factor. If mRNA molecules are produced and inactivated with a 30 min half-life, the amount of labeled molecules associated with the polyribosomes would approach a plateau with a half-time of 30 min.

The appearance of mRNA on polyribosomes during the time that the cell-produced factor is turning over rapidly is shown in Fig. 7. mRNA is measured by comparing the EDTA-sensitive and -resistant labeled material in the polyribosome region of sucrose gradients. Similar results were obtained by banding gluteraldehyde-fixed ribosomes on cesium chloride. The radioactivity in mRNA rises linearly during the period of measurement. No suggestion of a plateau is observed as would be the case if mRNA is synthesized and inactivated with a 30-min half-life.

The labeling of mRNA was accomplished by adding a very small amount of high specific activity ³H-uridine (2.5×10^{-4} μmole/ml, 20 Ci/mmole) at 25 min after infection to cells previously treated with low actinomycin to suppress ribosomal RNA labeling. This amount of uridine is fully incorporated into the cell within 15 min, and no further net increase in labeling of macromolecules takes place (R. Leibowitz and S. Penman, *unpublished observations*). Thus, by the time that the measurement of mRNA begins, both the nucleotide pools and unstable RNA molecules have reached their maximum degree

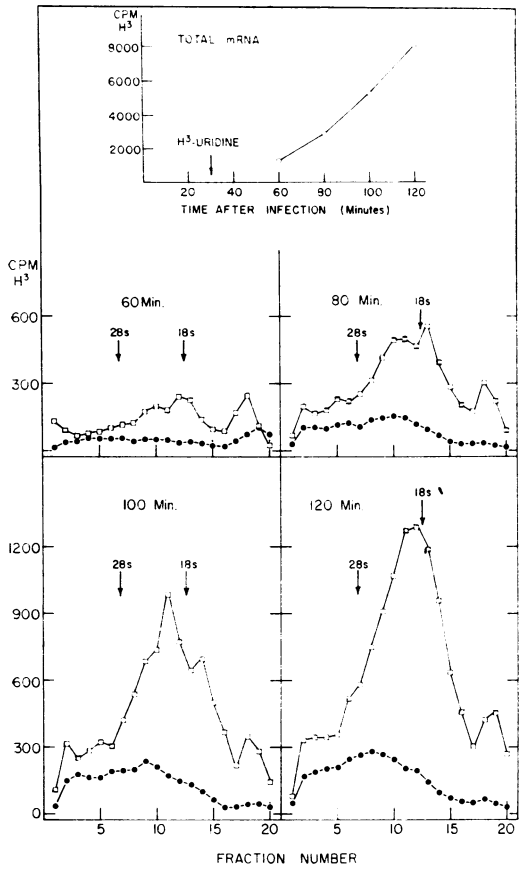

FIG. 7. *Kinetics of labeling of mRNA in virus-infected cells. Cells (1.3 × 10⁸) were infected in the presence of 31 mM guanidine. The cells were treated with 0.04 μg of actinomycin per ml from the start of the infection and ³H-uridine was added at 25 min after infection. Sample (3.2 × 10⁷) cells were removed and rapidly chilled at 60, 80, 100, and 120 min. RNA from polysomes and from co-sedimenting, EDTA-resistant structures was prepared from each sample as described in the legend to Fig. 4. The SDS-sucrose gradient sedimentation profiles of the RNA obtained at the various times after infection are shown in the lower four figures above. The time course of the accumulation of radioactivity in polysome-associated mRNA minus the EDTA-resistant fraction of each sample is shown in the top figure. Polysome RNA (—□—); EDTA-resistant RNA (—●—).*

of labeling and can only decline during the period of measurement. A declining nucleotide pool specific activity during the period of measurement could only favor the result opposite to the one obtained, i.e., a plateau in the amount of labeled message associated with polyribosomes.

DISCUSSION

The mechanism of virus-induced inhibition of host cell protein synthesis is subtle. No gross alterations in any of the major components of protein synthesis are observed. Yet, in the infected cell, ribosomes will translate virus-specific mRNA and fail to translate host mRNA even though it is apparently still present (24). A response occurs in some types of cells which maintains host protein synthesis for a considerable period after infection and which is inhibited by actinomycin.

Virus infection results in a decreased loading of ribosomes on host mRNA, as evidenced by a decreased rate of polyribosome sedimentation and a decreased number of ribosomes per mRNA molecule. These results suggest an inhibition of the process by which ribosomes associate with host mRNA and initiate polypeptide synthesis. Other possible explanations for a decreased loading of ribosomes on mRNA appear unlikely. The partial reloading of polyribosomes in low levels of cycloheximide also supports the hypothesis that the initiation of translation is inhibited.

The nature of the factor produced by the host cell which maintains protein synthesis remains to be elucidated. The results of the experiments shown in Fig. 7 indicate that the component which maintains the translation of host mRNA after virus infection is not new mRNA itself.

Since these experiments were performed, experimental results have been reported which show the inhibition of reticulocyte translation initiation by extracts from poliovirus-infected cells (10). The inhibitory agent has been shown to be double-stranded RNA (4). At present, it is not possible to relate these findings to the present work. The experiments reported here were all performed in the presence of guanidine, and the formation of double-stranded RNA under these conditions is problematic. Furthermore, the experiments reported here and in previous work (24) strongly suggest that the polio-produced inhibitor functions catalytically and results in an ever-increasing rate of turnover of the host-produced factor. It is difficult to relate this catalytic behavior to the inhibition by double-stranded RNA which would be expected to function stoichiometrically. It is also not clear, at present, that the initiation of hemoglobin synthesis in rabbit reticulocytes represents a general mechanism for mammalian cells. A fuller understanding of the mechanisms of inhibition of host cell protein synthesis will be possible when the initiation process for general cell protein synthesis can be studied in vitro.

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