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Selective Inhibition of Reovirus Ribonucleic Acid Synthesis by Cycloheximide

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Cycloheximide, at a concentration of 10 μg/ml, rapidly blocked protein synthesis in L cells infected with reovirus. When the drug was added before 5 hr postinfection, synthesis of both single- and double-stranded varieties of virus-specific ribonucleic acid (RNA), which normally commences between 6 and 7 hr after infection, was blocked. When the cycloheximide was added at 9 hr after infection, uptake of uridine-H^3 into RNA, for the succeeding 6 hr at least, was similar to that of an infected culture without the drug. This latter uptake of uridine-H^3 in the presence of cycloheximide was largely into single-stranded RNA, since double-stranded RNA synthesis was rapidly and markedly inhibited by the cycloheximide. Single-stranded RNA formed in the presence of cycloheximide was found not to be a precursor of viral progeny, double-stranded RNA. Synthesis of double-stranded RNA in the infected cell probably requires prior synthesis of a new protein, which has a rapid rate of turnover. The possibility that formation of single-stranded RNA is preceded by synthesis of a second new protein is discussed.

Approximately 7 hr after infection of L cells with reovirus, two species of virus-specific ribonucleic acid (RNA) can be detected (2). One species is double-stranded RNA, as Loh and Soergel (4) also found; it amounts to 25 to 40% of the total RNA made, and, judged by its chromatographic behavior on columns of methylated bovine albumin and by its sedimentation properties, it is viral progeny RNA. The remainder of the newly synthesized RNA is single-stranded. Part of this latter material is a messenger RNA (mRNA) specified by the viral genome; it is formed in the presence of sufficient actinomycin D to block synthesis of cellular mRNA (2); it is found in association with polyribosomes, and at least 40% of it will hybridize with denatured double-stranded viral RNA (6). Whether all the single-stranded RNA formed in infected cells is mRNA is still an open question. On the basis of preliminary experiments with puromycin, it has recently been suggested that at least two new proteins are involved in synthesis of virus-specific RNA (3).

In the present paper, we are concerned with the effect of cycloheximide, an inhibitor of protein synthesis, on the kinetics of RNA synthesis in reovirus-infected cells. When added at 5 hr after infection, cycloheximide prevented the synthesis of virus-specific RNA. When added later than 9 hr after infection, cycloheximide inhibited the synthesis of double-stranded RNA, but single-stranded RNA accumulated in the cells at an increased rate. The possibility that some of this single-stranded RNA may be a direct precursor of viral RNA is considered.

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MATERIALS AND METHODS

Cells and medium. Suspension cultures of L cells and media were used as described by Kudo and Graham (2).

Virus and procedure for infecting cultures. Reovirus, type 3, Dearing strain, was used as previously described (2). L cells from an exponentially growing culture were centrifuged and resuspended in fresh medium to which virus had been added to give a final concentration of approximately 2×10^6 cells per milliliter and a multiplicity of infection of 8 to 10 plaque-forming units (PFU) per cell. The cells were kept in suspension for 2 hr at room temperature to allow adsorption of the virus, were centrifuged, and were resuspended to a concentration of 5×10^5 cells per milliliter in fresh, prewarmed medium containing 0.5 μg of actinomycin D per ml. The culture was then placed at 37 C, and this moment was regarded as zero-time for the experiment. This procedure for infecting the culture was different from that employed earlier by Kudo and Graham (2).

Labeled compounds. Uridine-5-H^3 (25 c/mmole), uridine-2-C^{14} (25 mc/mmole), lysine-H^3 (2.5

c/mmole), and a C^{14}-labeled amino acid mixture were supplied by New England Nuclear Corp., Boston, Mass. The amino acid mixture is described by the company as a mixture of purified C^{14}-labeled amino acids in the same relative proportions found in a typical algal protein hydrolysate.

Prelabeling of cells with C^{14}. For some of the experiments to be described, the cellular RNA was labeled with C^{14} before infection. For this purpose, 0.5 μc of uridine-C^{14} and 5 to 10 μg/ml of thymidine were added to a 200-ml culture containing 5×10^5 cells per milliliter. The cells were allowed to grow at 37 C for 24 hr, centrifuged, and resuspended in fresh medium. After a further 24- to 48-hr growth period, during which time the cell concentration was maintained between 5×10^5 and 8×10^5 per milliliter, the C^{14} was entirely acid-insoluble and amounted to some 300 to 500 counts per min per 5×10^5 cells. This C^{14} marker was useful in one way in that its loss from infected cells denoted that some lysis was occurring. Its major utility will be described in the following section.

Extraction and analysis of RNA. The total RNA of cells was extracted with phenol-sodium dodecyl sulfate (SDS) at 60 C as previously described (2). Prior to sucrose gradient sedimentation analysis, the RNA, dissolved in LTM buffer [0.14 M LiCl, 0.01 M tris(hydroxymethyl)aminomethane, *p*H 7.6, and 0.001 M $MgCl_2$], was passed through a column of G-25 Sephadex gel, was precipitated from the effluent by addition of 2 volumes of ethyl alcohol, was redissolved in a small volume of LTM buffer, and was layered over a 5 to 20% linear gradient of sucrose. Sedimentation was at 36,000 rev/min for 3.5 hr in the SW39 head of a Spinco model L centrifuge. Fractions collected from the bottom of the tube into LTM buffer were analyzed for optical density at 260 mμ and then divided into two parts. Ribonuclease was added to one portion to a concentration of 5 μg/ml. After 30 min at 37 C, a drop of 0.5% bovine albumin solution and then an equal volume of cold 10% trichloroacetic acid were added to all samples. The precipitates were collected on membrane filters (Millipore Filter Corp., Bedford, Mass.), washed several times with cold 5% trichloroacetic acid, placed in scintillation vials, dried at 110 C, and covered with 2 ml of 1,4-bis-2-(5-phenyloxazolyl)-benzene-2,5-diphenyloxazole-toluene scintillation fluid. Radioactivity was determined in a model 4000 Scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Acid-insoluble radioactivity remaining after the digestion with ribonuclease was defined as double-stranded RNA.

The kinetics of incorporation of uridine-H^3 into single- and double-stranded RNA were determined as follows. Cells, prelabeled with C^{14} as described in the preceding section, were infected in the presence of actinomycin D and labeled with uridine-H^3 in the various ways to be described. Samples of 3 to 4 ml were withdrawn from the cultures, quick-frozen, and stored at −70 C until required. RNA was then extracted from each sample by the phenol-SDS procedure at 60 C. Extracted RNA was precipitated by addition of 3 volumes of ethyl alcohol, kept at −70 C for 18 hr, centrifuged, washed once with ethyl alcohol, and dissolved in 2 ml of LTM buffer. Measured amounts of this solution were mixed with an equal amount of cold 10% trichloroacetic acid, and the precipitates were filtered and assayed for C^{14} and H^3 to measure the total amount of newly synthesized RNA. Other measured volumes were treated at 37 C for 30 min with 5 μg of ribonuclease per ml prior to trichloroacetic acid precipitation and filtration to provide an estimate of the amount of double-stranded RNA. Since the cells had been labeled in their RNA with C^{14} before the experiment, the C^{14} contents of the samples could be used as the common denominator to normalize the amount of H^3 taken up. The newly synthesized fraction of RNA in each sample within an experiment is thus expressed as the ratio H^3/C^{14}, and, in this way, the H^3 contents of the various samples are directly comparable regardless of variable losses that may have been incurred during manipulation.

Acid-insoluble radioactivity. Incorporation of labeled nucleic acid precursors into the acid-insoluble constituents of cells was determined by removing a small sample of the culture into an equal quantity of ice-cold 10% trichloroacetic acid. The precipitate was collected 10 min later by filtration on a glass-fiber disc and washed with cold 5% trichloroacetic acid. The filter was put into a scintillation vial, and a drop of concentrated NH_4OH was placed near the center; it was dried at 110 C and covered with 2 ml of scintillation fluid, and its radioactivity was determined. NH_4OH reduced quenching of radioactivity, presumably by dissolving the excess of protein and distributing it throughout the filter.

To measure the incorporation of C^{14}-labeled amino acid mixture into protein, 1-ml samples were withdrawn from the cultures into 1 ml of 10% trichloroacetic acid and were heated at 100 C for 10 min. The resulting precipitates were washed three times with 5% trichloroacetic acid, dissolved in NH_4OH, transferred to glass filters, dried, and assayed for C^{14}.

RESULTS

Inhibition of protein synthesis by cycloheximide. To determine the concentration of cycloheximide required to inhibit protein synthesis, various concentrations of the drug were added to a series of cultures of L cells together with lysine-H^3. After incubation of the cultures for periods of 30 min and 3 hr, samples were withdrawn for assay of acid-insoluble H^3. As shown in Fig. 1, protein synthesis was inhibited to the extent of 85% in 30 min and 97% in 3 hr by 10 μg of cycloheximide per ml. Either 10 or 20 μg of drug per ml was used in the succeeding experiments.

An experiment was then carried out to find whether the inhibitory effect of cycloheximide on protein synthesis might be reversible. The uptake of C^{14}-labeled amino acids in a culture which had contained cycloheximide for 70 min was compared with that of a culture which had

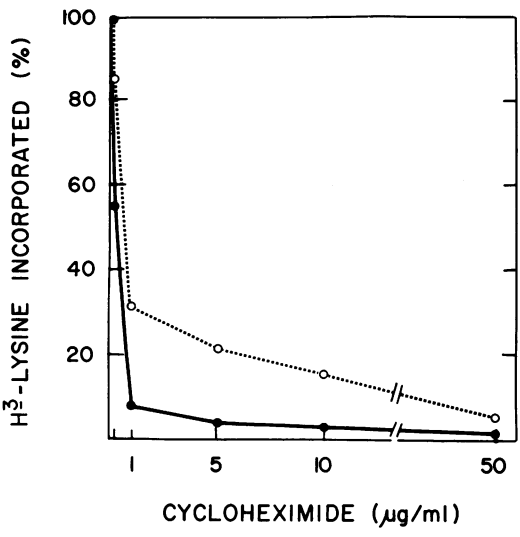

Fig. 1. *Inhibition of protein synthesis in L cells by different concentrations of cycloheximide. Cultures contained 5 × 10⁵ cells per milliliter. Lysine-H³, 0.8 μc/ml, was added to each at the same time as cycloheximide. A series of control cultures not treated with cycloheximide received a similar amount of lysine-H³. The points represent uptake of H³ into acid-insoluble material after 30 min (○) with cycloheximide and after 3 hr (●) with cycloheximide, as a percentage of the H³ incorporated into the respective control cultures.*

contained no cycloheximide and of one which contained cycloheximide throughout the period of incorporation. As shown in Fig. 2, when the cycloheximide was removed after a 70-min period of inhibition, protein synthesis resumed at a rate somewhat less than normal after a lag of 30 min. This reduced rate of protein synthesis after removal of the inhibitor is not considered to be significant; it could have resulted from some loss of cells during centrifugation and from a small error in adding the second quantity of labeled amino acids.

Figure 3 illustrates the effect of cycloheximide on the kinetics of protein synthesis in reovirus-infected cells. A culture was divided into two portions at 8 hr after infection when synthesis of virus-specific components was well under way (2). Lysine-*H*³ was added to one part, and cycloheximide and lysine-*H*³, to the other. It is seen that, within 30 min of its addition, cycloheximide almost completely blocked further incorporation of H³ into the acid-insoluble constituents of the infected cells.

Effect of cycloheximide on RNA synthesis in infected cells. It was previously shown (3) that puromycin added 5 hr after infection prevented the synthesis of virus-specific RNA, but, when added at 7 hr, it permitted some of this RNA to form. At the beginning of the present work, a series of similar experiments was carried out with cycloheximide. These experiments will not be given in detail here since the technique used has already been fully described for the puromycin experiments. Cycloheximide gave results similar to those with puromycin: when it was added 7 hr or later after infection, virus-specific RNA was synthesized, but none was detected when the inhibitor was added at 5 hr postinfection or earlier.

Selective inhibition of double-stranded RNA synthesis by cycloheximide. Virus-specific RNA, which is first detected at 6 to 7 hr after infection,

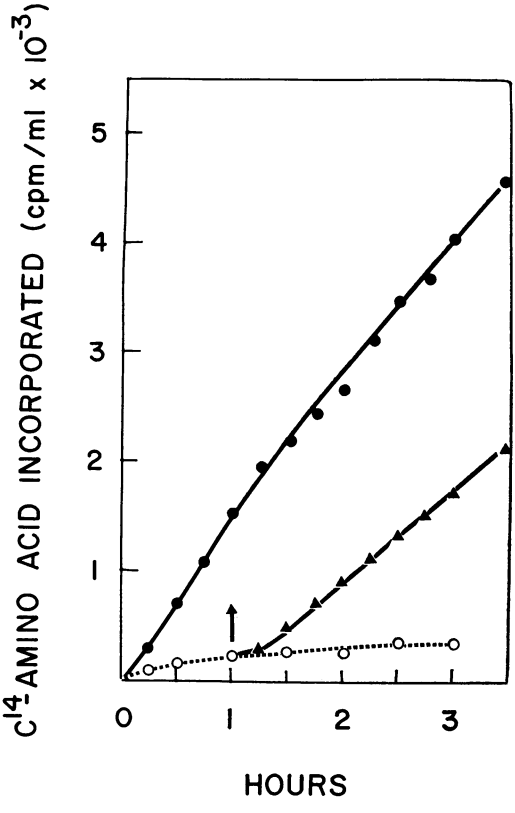

Fig. 2. *Reversibility of the cycloheximide inhibition of protein synthesis in L cells. Curves show the uptake of C¹⁴ into acid-insoluble constituents of three cultures under the following sets of conditions. (●) No cycloheximide, C¹⁴-labeled amino acid mixture added, 0.2 μc/ml; (○) 20 μg of cycloheximide per ml and, after 10 min, 0.2 μc of amino acid mixture per ml; (▲) 20 μg of cycloheximide per ml and, after 10 min, 0.2 μc of amino acid mixture per ml. Then, 60 min later, the cells were sedimented, washed, and suspended in medium containing 0.2 μc of amino acid mixture per ml and no cycloheximide.*

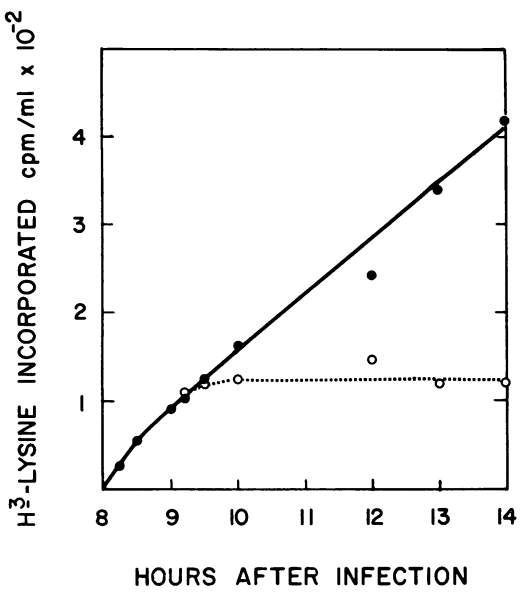

FIG. 3. *Inhibition of protein synthesis by cycloheximide in reovirus-infected L cells. The curves show the uptake of H^3 into an acid-insoluble form in two cultures to which lysine-H^3 was added 8 hr after infection. (●) No cycloheximide; (○) 10 μg of cycloheximide per ml added 9 hr after infection.*

is comprised of both single- and double-stranded species (2). To determine whether cycloheximide would selectively depress the formation of double-stranded RNA, as did puromycin (3), infected cultures were labeled with uridine-C^{14} after the addition of cycloheximide at 9 hr and between 9.5 and 11 hr and between 11.5 and 12.5 hr postinfection. Immediately after the period of labeling, RNA was extracted from each culture and analyzed by sucrose gradient sedimentation. The results, together with those of the appropriate control cultures to which no cycloheximide had been added, are shown in Fig. 4.

Figure 4a shows that in an uninfected culture actinomycin D suppressed the entry of C^{14} into all RNA but the 4*S* fraction when labeled at a time corresponding to 9.5 to 11 hr after infection. In an infected culture, there was considerable synthesis of RNA during the same period as shown by the broken line in Fig. 4b. The RNA formed in infected cells contained a peak of ribonuclease-resistant material, viral progeny RNA, sedimenting between 8 and 20*S*. There was a broad spectrum of single-stranded RNA, represented by the difference between the broken and dotted lines (Fig. 4b), which, in addition to the RNA that normally appeared under the 4*S* peak, comprised material sedimenting between 4 and 35*S* approximately. These results are in accord with previous work (2, 3). When cycloheximide was added 9 hr after infection, much less double-stranded RNA was made between 9.5 and 11 hr (Fig. 4c) than in the control culture, and practically none was detectable between 11.5 and 12.5 hr (Fig. 4d). In both the latter cases, a good deal of single-stranded RNA was formed. In fact, when the amount of C^{14} contained in single-stranded RNA in each of the gradients of Fig. 4b and 4c was normalized to a uniform optical density, it appeared that more single-stranded RNA was made in infected cells treated with cycloheximide than with no drug present. Moreover, when the C^{14} contained in double-stranded RNA in each fraction of the gradients (Fig. 4b and 4c) was subtracted from the total C^{14}, the resulting sedimentation patterns for single-stranded RNA synthesized in the presence of cycloheximide were similar to those for single-stranded RNA formed in its absence. This latter result suggests that no new type of single-stranded RNA is made in infected cells under the influence of cycloheximide. The same conclusion has been drawn from similar experiments with puromycin.

The kinetics of synthesis of virus-specific RNA in cycloheximide-treated cells were then studied in more detail in the following two experiments. The first experiment was similar in plan to that just described. Cycloheximide was added to a series of cultures at 8.85 hr after infection. Each culture was labeled with uridine-H^3 for 1 hr at various later times; the RNA was extracted with phenol and analyzed for total RNA-H^3 and double-stranded RNA-H^3. The results (Table 1) extend some of the conclusions tentatively drawn from the previous experiment. The overall rates of synthesis of virus-specific RNA during the 1-hr intervals were similar in the cycloheximide-treated and untreated cultures. The amounts of double-stranded RNA formed during the several intervals were fairly constant and averaged about 32% of the total virus-specific RNA synthesized in untreated cells. However, the addition of cycloheximide at 9 hr reduced the rate of double-stranded RNA formation by about 50% during the succeeding hour and to approximately 15% of normal during each of the next 3 hr.

The second experiment was set up in a somewhat different way. Cycloheximide was added to one of two cultures 8.75 hr after they had been infected; uridine-H^3 was then added to both cultures at 9 hr, and the time course of H^3 incorporation into total RNA and double-stranded

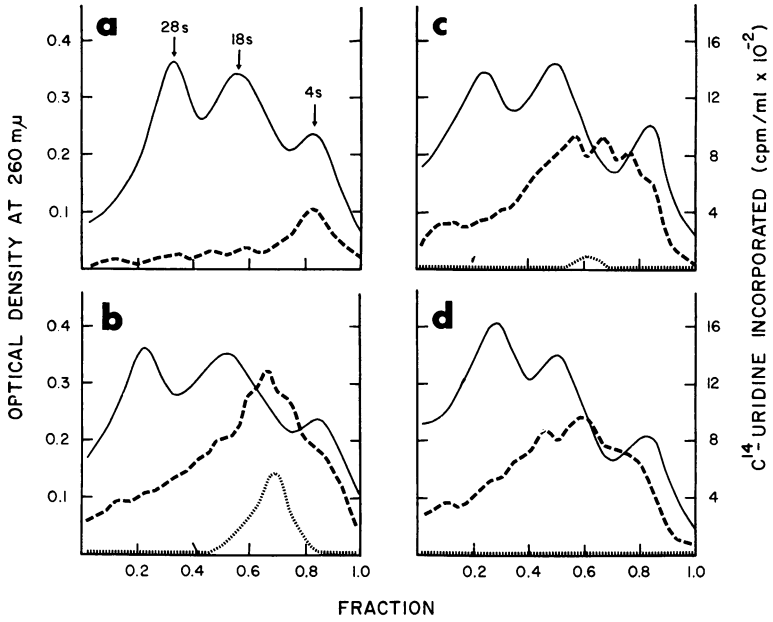

FIG. 4. *Sucrose gradient sedimentation analysis of RNA formed in infected cells in presence and absence of cycloheximide. (a) Uninfected control culture, no cycloheximide, labeled between 9.5 and 11 hr after commencement of the experiment with 0.05 μc of uridine-C¹⁴ per ml; (b) infected, no cycloheximide, labeled between 9.5 and 11 hr postinfection; (c) infected, 10 μg of cycloheximide per ml at 9 hr and labeled between 9.5 and 11 hr postinfection; (d) infected, cycloheximide added at 9 hr and labeled between 11.5 and 12.5 hr postinfection. RNA was extracted from each culture immediately after the period of labeling and analyzed. (Solid line refers to optical density at 260 mμ, dashed line to total C¹⁴, and dotted line to ribonuclease resistant C¹⁴).*

TABLE 1. *Rate of synthesis of virus-specific RNA at different times after the addition of cycloheximide*

RNA fraction	Cycloheximide[a]	Amt H^3 incorporated into virus-specific RNA[b]			
		9–10 hr[c]	10–11 hr	1–12 hr	12–13 hr
	μg/ml				
Total RNA	None	12.3	10.4	9.2	9.4
	20	8.2	9.0	9.4	9.6
Double-stranded RNA	None	4.6	3.2	2.5	2.8
	20	1.6	0.4	0.6	0.4
Double-stranded RNA, as per cent of the total RNA	None	37	31	27	30
	20	19	4.4	6.4	4.2

[a] Cycloheximide was added at 8.85 hr after infection.

[b] Expressed as H^3/C^{14} in the RNA fraction, the cells having been prelabeled with uridine-C^{14}. Results for the incorporation of H^3 have been corrected for the amount of H^3 taken up into parallel uninfected control cultures.

[c] Period of time after infection during which culture was labeled.

RNA was determined for each culture. The results (Fig. 5a) indicate that cycloheximide had little effect on the synthesis of virus-specific RNA up to 15 hr after infection. Uptake of H^3 into uninfected control cultures was negligible whether or not they were treated with cycloheximide, as the lower two curves of Fig. 5a show. Figure 5b represents the incorporation of H^3 into single- and double-stranded RNA in the infected cultures, and the results are consistent with those of the two preceding experiments. Cycloheximide markedly inhibited further synthesis of double-stranded RNA, but the amount of single-stranded RNA increased sufficiently

to keep the overall rate of virus-specific RNA synthesis similar to that in the culture without the drug. When cycloheximide was added at 7 hr after infection in this type of experiment, the amount of virus-specific RNA formed was less than that in an untreated, infected control culture. Discussion of these results will be deferred until later but one obvious interpretation may be pointed out here; namely, that part of the single-stranded RNA is a direct precursor of the double-stranded RNA and that cycloheximide, without preventing its continuing synthesis, blocks its further entry into the double-stranded form. This possibility has led to an experiment to be presented in a later section.

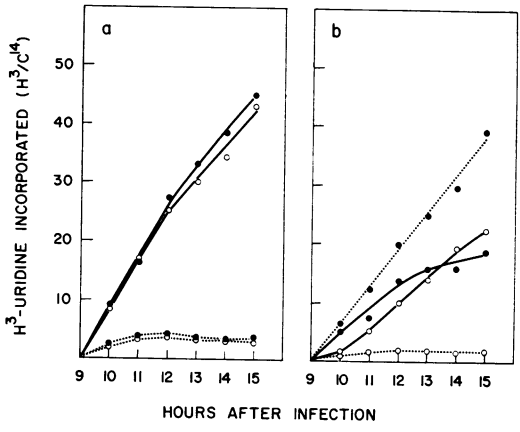

FIG. 5. *Effect of cycloheximide on the kinetics of incorporation of uridine-H^3 into virus-specific RNA. Cells were prelabeled with uridine-C^{14}. At 8.25 hr after infection, an additional 2 μg of actinomycin D per ml was added to all cultures and 2.0 μc of uridine-H^3 per ml was added at 9 hr. (a) Incorporation of H^3 into the total RNA of infected (solid line) and uninfected cells (broken line); (●) no cycloheximide; (○) 10 μg of cycloheximide per ml added at 8.75 hr. (b) Incorporation of H^3 into single-stranded RNA (●) and double-stranded RNA (○); no cycloheximide (solid line); with cycloheximide (broken line). Additional actinomycin D was added at 8.25 hr after infection to reduce synthesis of cellular RNA almost to zero; when added at this late time, the higher concentration of antibiotic had no effect on synthesis of virus-specific RNA (2).*

Effect of time of addition of cycloheximide on synthesis of double-stranded RNA. Whereas cycloheximide added 9 hr after infection markedly inhibited further synthesis of double-stranded RNA, the question was posed whether the drug would be equally efficient in this respect if added still later in the infectious cycle. In an experiment designed to answer this question, cycloheximide was added to samples of a culture taken at different times after infection. Uridine-H^3 was added to each sample 10 min after the cycloheximide. The RNA was extracted from each small culture 2 hr later and assayed for its content of H^3 and double-stranded RNA-H^3 (Table 2). When added at any time between 9 and 14 hr after infection, cycloheximide reduced the amount of double-stranded RNA formed during the succeeding 2 hr to 25 to 35% of that made in absence of the drug.

Reversibility of inhibition of double-stranded RNA synthesis. To have some assurance that the inhibitory effects being studied were due to some more or less direct action of cycloheximide rather than to some extensive degradative change induced in the cells by the drug, the reversibility of the double-stranded RNA inhibition was examined. The uptake of uridine-H^3 into double-stranded RNA was followed in a culture to

TABLE 2. *Effect of cycloheximide added at various times after infection on the formation of double-stranded RNA*[a]

RNA fraction	Cycloheximide[b]	Amt H^3 incorporated into virus-specific RNA[c]				
		9–11 hr[d]	10–12 hr	11–13 hr	13–15 hr	14–16 hr
	μg/ml					
Total RNA	None	3.1	2.7	2.9	1.9	1.7
	20	2.9	2.4	2.7	3.0	3.1
Double-stranded RNA	None	0.8	0.9	0.8	0.7	0.6
	20	0.3	0.2	0.2	0.4	0.3
Double-stranded RNA, as per cent of the total RNA	None	26	32	28	36	35
	20	10	8.4	7.4	13	9.7

[a] In this experiment an extra amount of actinomycin D, 2.0 μg/ml, was added 8.25 hr after infection.
[b] Cycloheximide was added 10 min before the addition of 2.0 μc of uridine-H^3 per ml.
[c] Expressed as H^3/C^{14} in the RNA fraction, the cells having been prelabeled with uridine-C^{14}. Results for incorporation of H^3 have been corrected for the amount of H^3 taken up into parallel uninfected control cultures.
[d] Period of time after infection during which culture was labeled.

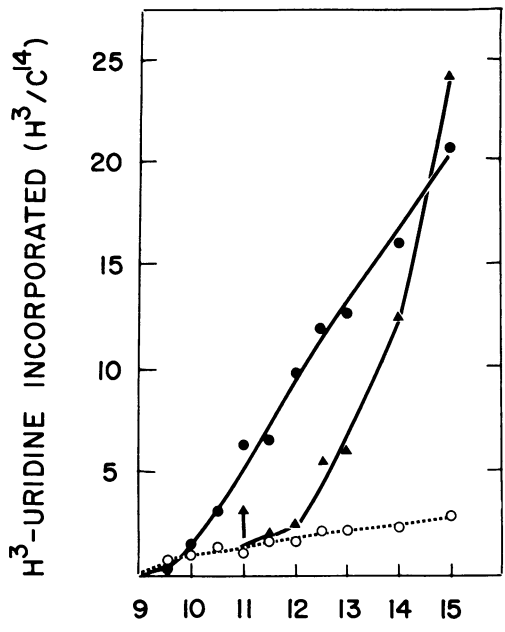

FIG. 6. *Reversibility of the cycloheximide inhibition of double-stranded RNA synthesis in infected cells. The curves show the uptake of H^3 into double-stranded RNA of three infected cultures. The cells had been prelabeled with uridine-C^{14}. At 8.25 hr after infection, additional actinomycin D, 2.0 μg/ml, was added to the cultures. (●) No cycloheximide, 2.0 μc of uridine-H^3 per ml added at 9 hr; (○) 20 μg of cycloheximide per ml added at 8.75 hr, uridine-H^3 added at 9 hr; (▲) 20 μg of cycloheximide per ml added at 8.75 hr after infection, 2.0 μc of uridine-H^3 per ml added at 9 hr; 2 hr later the cells were sedimented, washed, and suspended in medium containing 2.0 μc of uridine-H^3 per ml and no cycloheximide.*

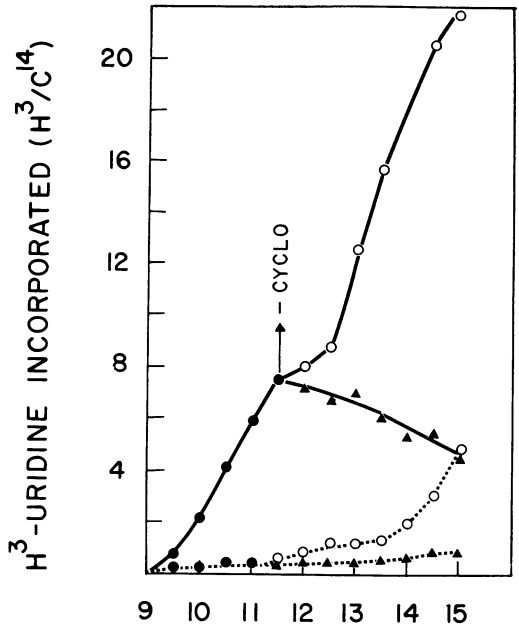

FIG. 7. *Fate of virus-specific single-strandep RNA formed in presence of cycloheximide upon removal of the drug. L cells prelabeled with uridine-C^{14} were infected in presence of 0.5 μg of actinomycin D per ml. At 8.5 hr after infection, an additional 2 μg of actinomycin D per ml was added. At 8.83 hr, 20 μg of cycloheximide per ml was added, followed by 1.7 μc of uridine-H^3 per ml at 9 hr. After 1 hr, at 10 hr after infection, the culture was centrifuged, and cells were washed once with warm medium and resuspended in warm medium containing 10 μg of cycloheximide per ml and 1.0 μg of actinomycin D per ml. At 11.5 hr, the culture was divided into two equal portions. To one portion, 50 μg each of unlabeled cytidine and uridine per ml were added, the culture was centrifuged, and cells were washed once with warm medium containing the same concentrations of uridine and cytidine and then resuspended to the original cell density in warm medium containing 50 μg of cytidine and uridine per ml. The second portion was centrifuged and the cells were washed once with warm medium and then resuspended in warm medium containing 1.7 μc of uridine-H^3 per ml. At 30-min intervals throughout the experiment, 3-ml samples were withdrawn and the RNA was extracted from each and assayed for incorporation of H^3 into the total RNA and into double-stranded RNA. An uninfected control culture was treated in exactly the same manner throughout, and the small incorporation of H^3 into this culture was subtracted from the assays obtained with the RNA of the infected culture. The figure shows the uptake of H^3 into the total RNA (solid line) and double-stranded RNA (broken line) in the infected culture; (●) uptake of H^3 added at 9 hr, (▲) cycloheximide removed at 11.5 hr and chased with unlabeled uridine and cytidine, (○) uridine-H^3 added again at 11.5 hr.*

which cycloheximide was added at 8.75 hr and removed at 11 hr after infection. One control culture contained cycloheximide throughout the period of exposure to uridine-H^3, and a third culture was left without the drug. It is clear from the results in Fig. 6 that the inhibitory action of cycloheximide was fully reversible; there was a lag of approximately 1 hr after removal of the drug and then synthesis of double-stranded RNA resumed at a rate similar to that in the uninhibited control culture.

Question of single-stranded RNA precursor of double-stranded RNA. The possibility was raised earlier that part of the single-stranded RNA formed in infected cells might be a direct precursor of double-stranded viral RNA. In principle, this question should be answerable by exposing infected cells for a short interval to a labeled RNA

precursor (pulse) and then adding a large excess of unlabeled precursor (chase). A movement of label from single- to double-stranded RNA during the chase would indicate that the former was the precursor of the latter. In practice, this simple experiment has proven to be difficult, if not impossible, to interpret owing to continued incorporation of label into RNA from the acid-soluble precursor pools during the chase. The observation that cycloheximide inhibited synthesis of double-stranded RNA but not of single-stranded RNA and that the inhibition was reversible suggested the following modification of the pulse-chase type of experiment.

Infected cells were labeled with uridine-H^3 between 9 and 10 hr postinfection in the presence of cycloheximide. Most of the incorporated precursor was thus directed into single-stranded RNA. The cells were then washed to remove the precursor and were returned to medium containing cycloheximide to allow the cellular pools to be drained of labeled material into single-stranded RNA. The cells were again washed and placed in cycloheximide-free medium containing a large excess of unlabeled uridine and cytidine to permit double-stranded RNA synthesis to recommence in the absence of further incorporation of label from the pools. To show that the cells were still capable of synthesizing both single- and double-stranded RNA after removal of cycloheximide, uridine-H^3 was added to a part of the culture in place of the chase of unlabeled nucleosides. The rather complicated details of this experiment are given in the legend to Fig. 7 in which the results are shown.

After removal of cycloheximide, the cells were still clearly capable of synthesizing both single- and double-stranded RNA, the latter after a somewhat extended lag. Furthermore, there was no increase in labeled RNA after addition of chase, indicating that the pools had been effectively drained during the previous sojourn of the cells in cycloheximide. In fact, between 11.5 and 15 hr postinfection, during the chase period, the amount of H^3 in single-stranded RNA dropped by almost 50% with practically no concomitant increase in double-stranded RNA-H^3. These results would seem to eliminate the single-stranded RNA formed in infected cells, or at least that part made in the presence of cycloheximide, as a major precursor of double-stranded RNA.

DISCUSSION

Cycloheximide, an inhibitor of protein synthesis in L cells (1), blocked the later formation of virus-specific single- and double-stranded RNA when added during the first 5 hr after infection. When the drug was added at 9 hr or later, synthesis of single-stranded RNA was not inhibited, whereas that of double-stranded RNA was markedly reduced. It is concluded that new protein must be made in response to infection, prior to the formation of either single- or double-stranded RNA. Further, since the ability of the infected cell to produce double-stranded RNA decays rapidly after addition of cycloheximide, it is presumed that a new protein specifically involved in formation of this RNA has a rapid rate of turnover and must be made continuously. The lag in formation of double-stranded RNA when the cycloheximide block was removed (Fig. 6) would then be explained as the time required to synthesize more of this specific protein.

An alternative explanation for the inhibitory effects of cycloheximide can be made if it is assumed that the drug has some action other than its effect on protein synthesis; for example, it could combine with viral double-stranded RNA, either parental or progeny, and prevent it from acting as a template for its own replication, or it might effect a direct block in the action of a pre-existing cellular enzyme engaged in the synthesis of the RNA. This possibility is remote, particularly since puromycin, an inhibitor of protein synthesis quite different in action from cycloheximide (5, 7, 8), also selectively inhibits the synthesis of double-stranded RNA (3).

Under the action of cycloheximide added 9 hr or later after infection, single-stranded RNA accumulates in the infected cell at a more rapid rate than in untreated cells. Since double-stranded RNA synthesis is inhibited under these conditions to almost the same extent that the extra single-stranded RNA builds up, there is an obvious possibility of a precursor-product relationship between the two species of RNA. However, when the single-stranded RNA was labeled in cycloheximide-treated cells, when the pools were allowed to drain out, and when a chase of unlabeled precursor was then added after removal of the cycloheximide (Fig. 7), the labeled single-stranded RNA decreased by some 50%, but none of the label appeared in the double-stranded RNA. These results provide strong evidence that the accumulated single-stranded RNA is not a precursor of the double-stranded RNA.

The sedimentation pattern of this single-stranded RNA (Fig. 4 and description thereof) is similar to that of single-stranded RNA formed in the absence of cycloheximide during the same period after infection [see also Kudo and Graham (3)]; the drug does not lead to the selective buildup of a specific class of single-stranded RNA. Moreover, at least part of the single-

stranded RNA in infected cells is associated with polyribosomes and has the properties of a mRNA (6). Thus, it is likely that the single-stranded RNA which piles up in cycloheximide-treated cells is a virus-specified mRNA and that the turnover it normally undergoes (Fig. 7) is prevented by the drug. This possibility is under study.

As a result of puromycin inhibition experiments, it was recently suggested (3) that synthesis of virus-specific single-stranded RNA required the prior synthesis of a second new protein, and the present results may be interpreted in the same way. However, it is still arguable that the only new protein whose formation has been shown to be obligatory is that involved in synthesis of double-stranded RNA. Thus, single-stranded RNA is first detected at approximately 7 hr after infection at the same time as double-stranded RNA, and its rate of synthesis can be markedly inhibited at that time by addition of puromycin or cycloheximide. By 9 hr after infection, neither puromycin nor cycloheximide inhibits the formation of single-stranded RNA. If this RNA is transcribed from viral progeny RNA, as seems likely, the transcription could be carried out by a pre-existing cellular enzyme. The effect of inhibitors of protein synthesis could then be explained on the assumption that a pool of free double-stranded RNA template which begins to form at 7 hr achieves its maximal size by 9 hr after infection. By this time, synthesis of new double-stranded RNA would be normally balanced by withdrawal of the RNA into virions; treatment with cycloheximide or puromycin would prevent further maturation and would rapidly inhibit further viral RNA formation owing to the decay of the specific protein involved, but the pool of free template would remain and permit the transcription of single-stranded RNA to go on at the normal rate. At least some of the assumptions invoked in this explanation are open to test.

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