SELECTIVE INHIBITION OF REOVIRUS INDUCED RNA IN L CELLS

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We have recently described two species of viral-specific RNA that begin to form approximately 7 hours after infection of L cells with reovirus (Kudo and Graham, 1965). One of the new RNA species was heterogenous in sedimentation properties, sensitive to low concentrations of RNase and undoubtedly single-stranded. The other species was RNase resistant and was probably the double-stranded progeny RNA. The relationship between these two RNAs is not yet known. In the present paper it is shown that puromycin, an inhibitor of protein synthesis, blocks the formation of both types of RNA when added early in the latent period. When added 9 hours post-infection, the antibiotic inhibits the synthesis of double-stranded RNA, but single-stranded RNA continues to be made.

METHODS

The methods for growth of L cells in suspension, infection with reovirus, extraction of RNA at 60°C with phenol-SDS,purification of extracted RNA by Sephadex column chromatography, and sucrose density gradient analysis have been fully described (Kudo and Graham, 1965). We are indebted to Dr. R. B. Anderson of Merck Institute of Therapeutic Research for providing the actinomycin D.

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RESULTS

Effect of puromycin on the synthesis of protein in infected cells. Preliminary experiments showed that 50 μgm per ml of puromycin was required to prevent incorporation of lysine-H^3 into the acid-insoluble constituents of normal L cells. Synthesis of protein in infected cells was totally inhibited within 30 minutes of addition of the puromycin.

Effect of puromycin on the synthesis of viral specific RNA. When puromycin was added 2 hours after infection, uridine-C^{14} could

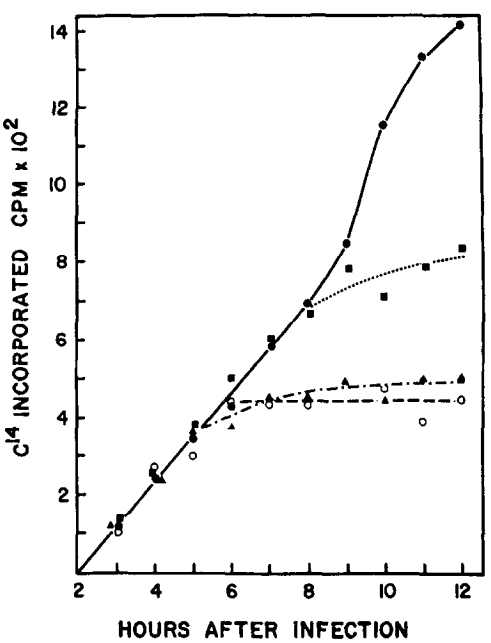

Figure 1. Effect of puromycin added 2 hours after infection on uptake of uridine-C^{14}. Actinomycin D, at a concentration of 0.5 μ gm per ml, was added to four suspension cultures of L cells each containing 5 x 10^5 cells per ml. After 1 hour, two of the cultures were infected with reovirus at a multiplicity of 8. Uridine-C^{14} (0.025 μ c per ml) was added to all four cultures 2 hours after infection. Immediately afterwards puromycin was added to one infected and one uninfected culture at a concentration of 50 μ gm per ml. At intervals, 2 ml samples were removed from each culture, an equal amount of cold 10% TCA was added to them and the precipitate was filtered and assayed for C^{14} with a Tricarb Liquid Scintillation Spectrometer. (●——●) infected culture; (■——■) uninfected culture; (○——○) infected culture plus puromycin; (▲——▲) uninfected culture plus puromycin.

be incorporated for a further 4 hours (Figure 1). The kinetics of incorporation in infected cells were, however, similar to those for an uninfected control culture to which puromycin had been added. Most of this newly synthesized nucleic acid is 4S RNA (Kudo and Graham, 1965). In the absence of puromycin, nucleic acid synthesis in infected cells continued until at least 12 hours, while in uninfected cells the rate of nucleic acid synthesis started to decrease from 7 hours after addition of the uridine-C^{14}.

The situation was different when puromycin was added 9 hours post-infection (Figure 2). Uninfected cells incorporated uridine-C^{14} slowly in the presence or absence of puromycin. Infected cells took up the uridine-C^{14} to a markedly greater extent than did these controls, even in the presence of puromycin. Other such experiments indicated that some viral-specific RNA continued to be formed when puromycin was added 7 hours post-infection but not when added at 5 hours.

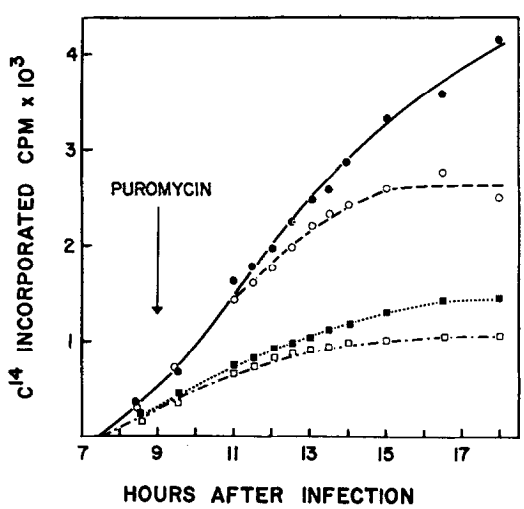

Figure 2. Effect of puromycin added 9 hours after infection on uptake of uridine-C^{14}. The design of this experiment was the same as that of Figure I except that uridine-C^{14} was added at 7.5 hours and puromycin at 9 hours after infection. (●——●) infected culture; (■——■) uninfected culture; (○——○) infected culture plus puromycin; (□——□) uninfected culture plus puromycin.

The following experiment was done to determine the nature of the RNA made after addition of puromycin at 9 hours post-infection. Cell cultures were labeled with uridine-C^{14} between 11 and 13 hours after infection and the RNA was then extracted and analyzed by sucrose gradient sedimentation. Each resulting fraction was assayed

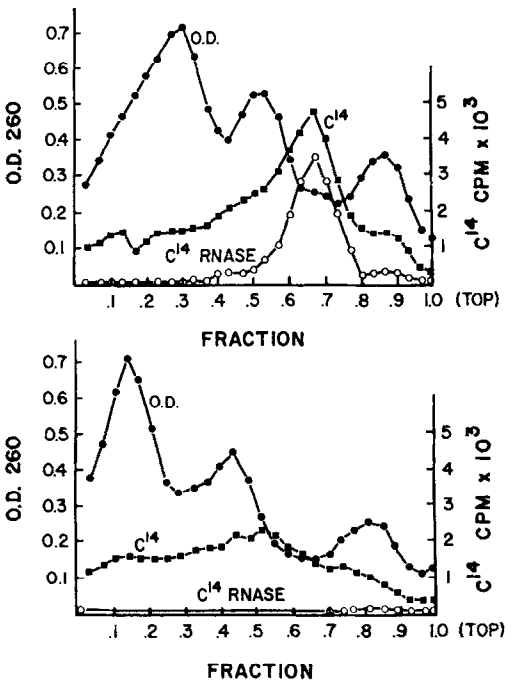

Figure 3. Effect of puromycin, added 9 hours after infection on the synthesis of RNA. Two 200 ml cultures of L cells (5 x 10^5 per ml) were set up to contain 0.5 μ gm of actinomycin D per ml. One hour later both cultures were infected with reovirus at a multiplicity of 8. At 9 hours after infection puromycin was added to one culture to a concentration of 50 μ gm per ml. Uridine-C^{14} was added (0.025 μ c per ml) to each culture at 11 hours and at 13 hours the two cultures were centrifuged and RNA extracted from the cells by the phenol-SDS technique (Kudo and Graham, 1965). Each sample of RNA was centrifuged through a linear 5 to 20% sucrose gradient in the SW39 rotor of a Spinco Model L centrifuge for 3.5 hours at 37,5000 rpm. Three drop samples were collected from the bottom of the centrifuge tube into 1 ml of 0.14M NaCl and the fractions were assayed for optical density at 260 mμ. Each fraction was then divided in two and RNAse was added to one portion to a concentration of 2 μ gm per ml. After 0.5 hours at 37°C a drop of 0.1% bovine serum albumen was added to each tube in the cold followed by 2 ml of cold 5% TCA_{14} The precipitates were filtered and assayed for C^{14}. C^{14} represents the total incorporated C^{14}, C^{14} RNAse represents the ribonuclease resistant RNA fraction. Top panel, no puromycin: bottom panel, puromycin added.

for acid-insoluble C^{14} before and after the action of RNase. RNA digested by the enzyme is defined as single-stranded, enzyme-resistant RNA as double-stranded. Synthesis of both single- and double-stranded RNA was observed in the absence of puromycin (Figure 3, upper panel). In the presence of puromycin only single-stranded RNA was formed (Figure 3, lower panel). Control experiments carried out with uninfected cells in the presence of actinomycin D showed incorporation of C^{14} into the 4S fraction only (Kudo and Graham, 1965). When the C^{14} of the double-stranded RNA peak (upper panel) was subtracted from the total RNA-C^{14}, to determine the distribution of single-stranded RNA-C^{14} in the uninhibited culture, the sedimentation patterns of single-stranded RNA synthesized in both cultures were found to be similar. Thus, puromycin added 9 hours after infection largely suppressed synthesis of double-stranded RNA by 11 hours, but formation of the various single-stranded RNA species continued. Further, when the C^{14} values for single-stranded RNA in the two gradients were summed and ratios calculated against the total optical densities in the respective gradients, it turned out that as much single-stranded RNA was synthesized in the puromycin treated cells as in the control cells.

DISCUSSION

Synthesis of viral specific RNA in L cells commences at approximately 7 hours after infection and this new RNA is composed of single- and double-stranded species. When puromycin is added at 9 hours after infection single-stranded RNA continues to be formed at almost the normal rate for another 4 hours. Synthesis of viral specific RNA does not occur if the puromycin is added before 5 hours post-infection. Since puromycin blocks protein synthesis in infected cells, the results are interpreted to mean

that synthesis of viral specific, single-stranded RNA depends on the formation of new protein(s) between 5 and 9 hours after infection.

Double-stranded RNA synthesis is normally well under way by 9 hours post-infection but it is markedly inhibited following addition of puromycin at 9 hours. This observation suggests that synthesis of another new protein(s) is required for formation of double-stranded RNA and that this protein turns over rapidly in the cell. This hypothesis is under study.

ACKNOWLEDGEMENT

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REFERENCES

Kudo, H., and Graham, A. F., J. Bacteriol., 90, 936, 1965.