JOURNAL OF VIROLOGY, Feb., 1967, p. 24-35
Vol. 1, No. 1

Reovirus-directed Ribonucleic Acid Synthesis in Infected L Cells[1]

A. J. SHATKIN AND B. RADA[2]

Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

Received for publication 11 October 1966

Reovirus replication in L-929 mouse fibroblasts was unaffected by 0.5 μg of actinomycin per ml, a concentration which inhibited cell ribonucleic acid (RNA) synthesis by more than 90%. Under these conditions of selective inhibition, the formation of both single-stranded and double-stranded virus-specific RNA was detected beginning at 6 hr after infection. The purified double-stranded RNA was similar in size and base composition to virus RNA and presumably was incorporated into mature virus. The single-stranded RNA formed ribonuclease-resistant duplexes when annealed with denatured virus RNA but did not self-anneal, thus indicating that it includes copies of only one strand of the duplex. The single-stranded RNA was polyribosome-associated and may function as the virus messenger RNA. Production of both types of virus-induced RNA required protein synthesis 6 to 9 hr after infection. At later times in the infectious cycle, only double-stranded RNA synthesis was dependent on continued protein formation.

Reovirus (8, 18), like wound tumor virus (6, 38) and rice dwarf virus (22), contains helical, double-stranded RNA. As in many virus-cell systems, analysis of virus-directed RNA synthesis in reovirus-infected L cells is difficult, because cell RNA synthesis continues after infection and masks the viral processes (7). This complication was minimized in cells infected with other RNA viruses when it was observed that cell RNA synthesis could be completely suppressed with actinomycin without preventing virus production (26, 31). Similarly, L-cell RNA synthesis can be inhibited more than 90% by 0.5 μg of actinomycin per ml without affecting reovirus replication (16, 32), although a concentration of 2 μg/ml inhibits reovirus replication by 90% (9). Under the conditions of selective inhibition by actinomycin, the rate of RNA synthesis in infected cells increased 10- to 15-fold above the rate in uninfected cells. This virus-induced RNA has now been isolated and found to consist of both double-stranded and single-stranded species. The double-stranded RNA was similar in size and base composition to the helical RNA extracted from purified reovirus and presumably is the virus progeny RNA. The single-stranded RNA was transcribed from the duplex by a process which apparently involved strand selection. Its association with polyribosomes suggests that it functions as messenger RNA for enzymes and coat proteins of the virus.

[1] A portion of these results was presented at the Symposium on the Molecular Biology of Viruses, University of Alberta, Edmonton, Canada, 29 June 1966.

[2] World Health Organization Fellow. Permanent address: Institute of Virology, Czechoslovak Academy of Sciences, Bratislava, Czechoslovakia.

MATERIALS AND METHODS

Cells and virus. L-929 cells were grown in suspension culture in Eagle's medium (3) containing 5% fetal bovine serum. Stock virus was prepared in monolayer cultures by passage of an inoculum of American Type Culture Collection reovirus type 3, Abney Strain. For infection, cells were concentrated to 5×10^6 cells per milliliter in growth medium containing 2% serum, and virus was added at a multiplicity of about 100 plaque-forming units (PFU) per cell. After adsorption at 37 C for 2 hr, the cells were centrifuged, washed once with media to remove unadsorbed virus, and resuspended at a concentration of 5×10^5 cells per milliliter. Infectious virus was measured by plaque assay on L-cell monolayers (9).

Isolation, purification, and characterization of RNA. After exposure to radioactive uridine or phosphate-P^{32}, the cells were chilled, centrifuged, and washed with Eagle's medium at 4 C. The cells were resuspended at a density of 10^7 cells per milliliter in 0.01 M acetate buffer (*p*H 5.1) containing 0.1 M NaCl and 0.001 M $MgCl_2$. Sodium dodecyl sulfate (final concentration 0.34%) and an equal volume of water-saturated phenol were added. The cell suspension was then shaken for 5 min at 60 C and rapidly cooled to 4 C. After centrifugation to separate the phases,

the aqueous portion was re-extracted with phenol, and the RNA was precipitated from the aqueous extract at −20 C by adding 2 volumes of ethyl alcohol.

For sucrose density gradient centrifugation, RNA which had been passed through Sephadex G-25 and precipitated with ethyl alcohol was dissolved in acetate buffer (0.1 M NaCl, no $MgCl_2$) and was overlaid onto 15 to 30% linear sucrose gradients in the same solution. After centrifugation for 16 hr at 25,000 rev/min in the Spinco SW-25.1 rotor, samples were collected through a hole punctured in the bottom of the tube. The optical density of the effluent was continuously monitored. Alternate fractions of 0.5 ml were either precipitated directly with 5% perchloric acid or, before precipitation, were incubated for 30 min at 37 C with 2 μg of ribonuclease per ml in 0.01 M phosphate buffer (*p*H 7.4) and 0.15 M NaCl. Under these conditions of ribonuclease treatment, double-stranded reovirus RNA is protected against degradation to acid-soluble material (33). Samples were precipitated with perchloric acid and 2 mg of yeast RNA as carrier, and the precipitates were dissolved in dilute NH_4OH and plated for counting in a low-background gas-flow counter. In some experiments, samples were precipitated with 5% trichloroacetic acid, collected on membrane filters (Millipore Filter Corp., Bedford, Mass.), and counted in liquifluortoluene in a scintillation counter.

Methylated-albumin-Kieselguhr (MAK) column chromatography was performed as described previously (19). RNA was dissolved at a concentration of about 0.2 mg/ml in 0.05 M phosphate buffer (*p*H 6.8) containing 0.3 M NaCl and applied to a MAK column. The RNA was eluted at 35 C by applying a linear gradient of 0.3 to 1.6 M NaCl in phosphate buffer. Fractions were collected, and samples were acid-precipitated and counted before and after ribonuclease digestion as described above.

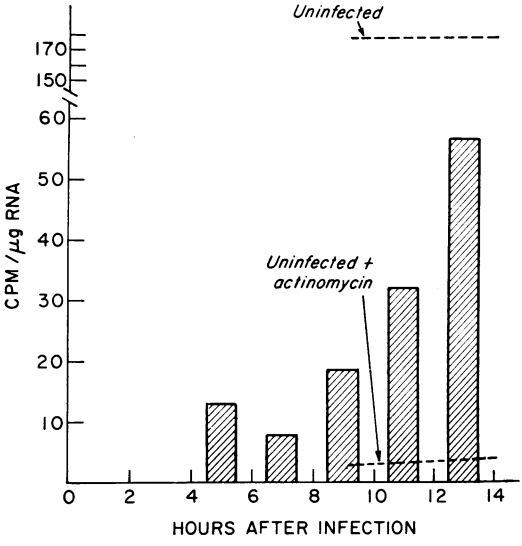

FIG. 1. *Stimulation of RNA synthesis in actinomycin-treated cells following reovirus infection. Infected cultures containing 0.5 μg of actinomycin per ml were exposed to uridine-2-C^{14} (2×10^{-6} M, specific activity = 24.4 μc/μmole) for 1 hr. The cells were then chilled, centrifuged, extracted two times with 5% perchloric acid at 4 C, and hydrolyzed with 0.3 N KOH at 37 C for 16 hr. Samples were plated for counting, and RNA was measured by the orcinol method (21). Uninfected cells were treated with actinomycin for 2 hr or longer before exposure to uridine-2-C^{14} for 1 hr.*

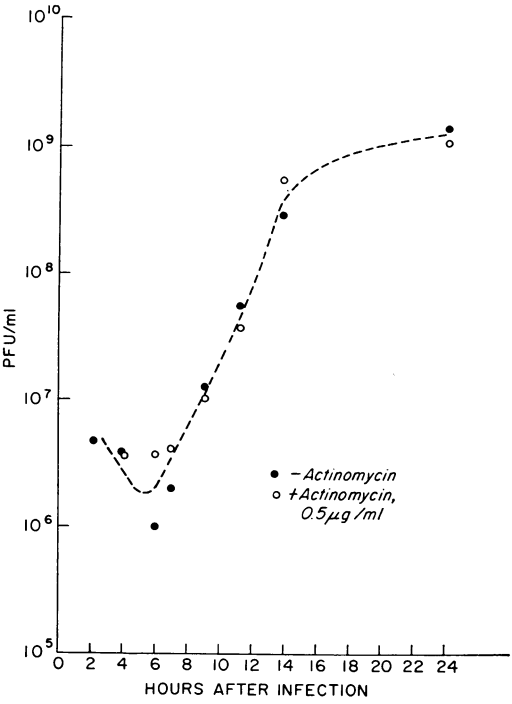

FIG. 2. *Time course of reovirus formation.*

Base composition analysis was performed by high-voltage paper electrophoresis of alkaline hydrolysates of P^{32}-labeled RNA in pyridine-acetate buffer at a *p*H of 3.5 (30).

Reovirus was purified from infected cells by genetron treatment and CsCl equilibrium density gradient centrifugation, and double-stranded RNA was extracted from virus with phenol (33). Before determination of its base composition, it was necessary to purify further the double-stranded virus RNA by centrifugation to equilibrium in a Cs_2SO_4 density gradient (33).

For annealing, radioactive RNA was dissolved in 0.5 ml of 0.3 M NaCl-0.005 M phosphate buffer (*p*H 7) and was mixed with 0.1 ml of 0.1 × standard saline citrate (SSC = 0.15 M NaCl and 0.015 M sodium citrate) containing denatured reovirus RNA. The virus RNA was denatured in 0.1 × SSC by heating for 10 min at 100 C, followed by quick cooling. The mixture was then placed in a water bath at 90 C and cooled to room temperature over a period of several hours.

The preparation of cytoplasmic fractions for sucrose density gradient analysis of polyribosome-asso-

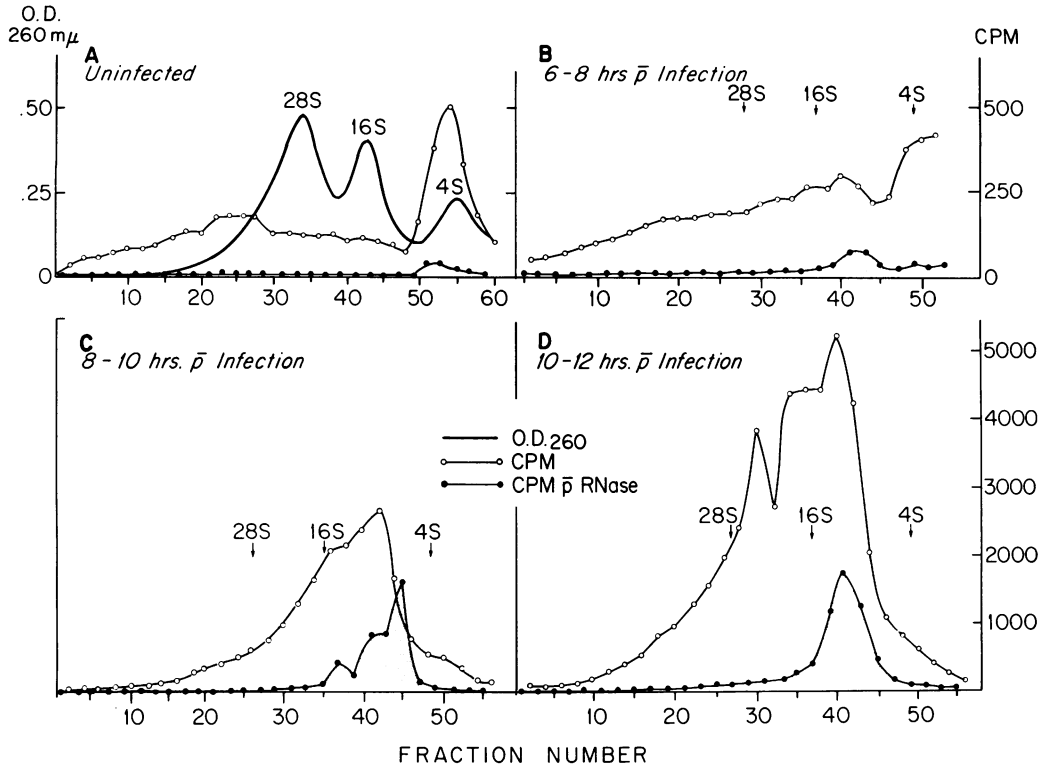

FIG. 3. *Sedimentation of newly formed RNA in sucrose density gradients. Cell cultures were incubated with uridine-2-C*^{14} *for the indicated time intervals, and the RNA was extracted. After centrifugation of the RNA through 15 to 30% sucrose density gradients (16 hr at 24,000 rev/min in Spinco SW-25.1 rotor), fractions of 0.5 ml were collected. Even-numbered tubes* (○) *were precipitated with 5% perchloric acid and counted. Odd-numbered fractions* (●) *were incubated with 2 μg of ribonuclease per ml in 0.01 M phosphate buffer (pH 7.4) and 0.15 M NaCl for 30 min at 37 C and then precipitated for counting. The abbreviation p̄ Infection means postinfection.*

ciated RNA has been described (25). Briefly, after incubation with uridine-2-C^{14}, cells were concentrated to 4 × 10^7 cells per milliliter in 2 ml of 0.01 M tris(hydroxymethyl)aminomethane buffer (*p*H 7.8) containing 0.01 M NaCl and 0.0015 M $MgCl_2$ and homogenized with a Dounce homogenizer at 4 C. Nuclei and unbroken cells were removed by low-speed centrifugation (800 × *g* for 2 min). The supernatant solution comprising the cytoplasmic fraction was made 0.25% with respect to sodium deoxycholate, layered onto 5 to 30% sucrose density gradients, and centrifuged for 2.5 hr at 24,000 rev/min in the Spinco SW-25.1 rotor.

RESULTS

Selective inhibition of cell RNA synthesis by actinomycin. Exposure of suspension cultures of uninfected L cells to 0.5 μg of actinomycin per ml for 2 hr or longer reduced the specific activity of RNA following 1 hr of incubation with uridine-2-C^{14} (specific activity = 24.4 μc/μmole, 2 × 10^{-6} M) from 180 counts per min per μg of RNA to 4 counts per min per μg (Fig. 1). After infection of L-cell cultures with a high multiplicity of reovirus, new infectious virus was detected 6 to 8 hr postinfection (PI), and the rise in titer was logarithmic for the next several hours. In contrast to cell RNA synthesis, the addition of 0.5 μg of actinomycin per ml at 2.5 hr PI did not alter the time course of maturation or reduce the final yield of about 2,000 PFU per cell (Fig. 2). In cultures treated with the antibiotic to suppress cell RNA synthesis and infected with reovirus, an increase in RNA formation was readily detected after virus infection (Fig. 1). At 12.5 to 13.5 hr PI, the rate of RNA synthesis in an infected culture was 14-fold greater than that observed in an uninfected, inhibited culture and one-third that of growing cells. Several properties including size, secondary structure, and chemical composition of this newly formed, virus-induced RNA were examined.

Size and secondary structure of newly formed RNA in actinomycin-treated, infected cells. Synthetic ribopolymers lose their capacity to

stimulate the incorporation of amino acids into polypeptides in vitro as the amount of ordered secondary structure which they contain increases (24, 34). Natural, double-stranded RNA extracted from rice dwarf virus (23) or reovirus (R. Krug, P. J. Gomatos, I. Tamm, and F. Lipmann, *unpublished data*) also functions poorly, if at all, as a template for in vitro protein synthesis. The inactivity of helical polymers is presumed to be due to their inability to bind effectively to ribosomes (36; R. Cukier and M. W. Nirenberg, *unpublished data*). These observations suggested that a necessary intermediate step in the replication of reovirus is the formation of a single-stranded RNA which can function as a messenger for viral protein synthesis. To examine this possibility, actinomycin-treated, infected cultures were exposed to uridine-2-C^{14} for 2 hr at various times during the infectious cycle. The RNA was then isolated and analyzed by sucrose density gradient centrifugation.

RNA extracted from an uninfected, actinomycin-treated culture contained 16*S* and 28*S* ribosomal species and 4*S* soluble RNA as shown by the optical density profile in Fig. 3A. Similar patterns were obtained from infected cell RNA preparations. When uninfected cells were incubated with 0.5 μg of actinomycin per ml for 3.5 hr followed by exposure to uridine-2-C^{14} for 2 hr, ribosomal RNA synthesis was almost completely inhibited. A low level of incorporation into a heterogeneous, larger RNA component and into 4*S* RNA persisted. The newly synthesized RNA was degraded to acid-soluble material during a 30-min incubation period with 2 μg of ribonuclease per ml.

In infected cells, RNA synthesis at 6 to 8 hr PI was increased 30% above that observed in uninfected, inhibited cultures (Fig. 3B). The RNA formed during this interval was also largely single-stranded as demonstrated by its susceptibility to digestion by ribonuclease. However, 9% of the newly synthesized RNA was nuclease-resistant (shaded area). Its sedimentation value of about 10 to 12*S* is the same as that for the double-stranded RNA that is extracted from purified reovirus. Double-stranded RNA isolated at later times in the infectious cycle also sediments at 10 to 12*S*. At 8 to 10 hr PI, the rate of RNA synthesis increased to fivefold that of uninfected cells (Fig. 3C). The newly formed RNA was heterogeneous and sedimented as a broad peak with a maximum at 12 to 16*S*. Of the total RNA synthesized during this interval, 23% was nuclease-resistant. A further stimulation in RNA synthesis to more than ten times the level in uninfected cells was observed at 10 to 12 hours PI, and the double-stranded RNA comprised 15% of the total RNA synthesized (Fig. 3D). Kudo and Graham (16) have also found that the major fraction of newly formed RNA in reovirus-infected, actinomycin-treated cells is single-stranded and sensitive to degradation by ribonuclease.

Column chromatography of RNA. Separation of single and double-stranded RNA was achieved by chromatography on MAK columns (19). Portions of the RNA preparations used for sucrose gradient analyses (Fig. 3) were also chromatographed on MAK columns, and the elution profiles are shown in Fig. 4. As seen in the optical density tracing in each panel, soluble RNA eluted at 0.4 to 0.5 M NaCl, and ribosomal RNA, at 0.9 to 1.1 M. Samples of each eluted fraction were tested for acid-precipitable radioactivity before and after incubation with ribonuclease. In agreement with the sucrose gradient analysis, the radioactivity pattern from uninfected cells included newly formed soluble RNA and a smaller quantity of material eluting at 1.0 to 1.2 M NaCl. Both components were digested to acid-soluble material by ribonuclease. The base composition of the larger material (expressed in moles of each nucleotide per 100 moles of nucleotides: cytidylic acid, 25.1; adenylic acid, 24.6; guanylic acid, 24.4; uridylic acid, 25.9) is intermediate between that of 16*S* ribosomal RNA and cell DNA (27, 29), suggesting that it may include cell messenger RNA (15).

RNA which was synthesized in infected cells 6 to 8 hr PI consisted of soluble RNA and a slightly increased amount of RNA eluting with the ribosomal RNA. Both were nuclease-sensitive. In addition, there was a component which was resistant to ribonuclease digestion and which eluted at 0.7 to 0.8 M NaCl. This double-stranded RNA was well separated from the virus-specific, single-stranded material which comprised the bulk of the newly formed RNA. Similar elution profiles were obtained with RNA preparations isolated from cells incubated 8 to 10 and 10 to 12 hours PI with uridine-2-C^{14}.

Base composition analysis. The finding that single-stranded RNA could be detected and isolated from actinomycin-treated, reovirus-infected cells provided an opportunity to analyze the composition of virus-induced RNA which was not incorporated into mature virus. RNA was extracted from antibiotic-treated cells after incubation 6 to 9 or 9 to 12 hr PI with radioactive phosphate-P^{32} (10^{-5} M phosphate, 7.5 μc/ml). Single- and double-stranded RNA were separated on a MAK column, and their base compositions were compared with RNA-P^{32}

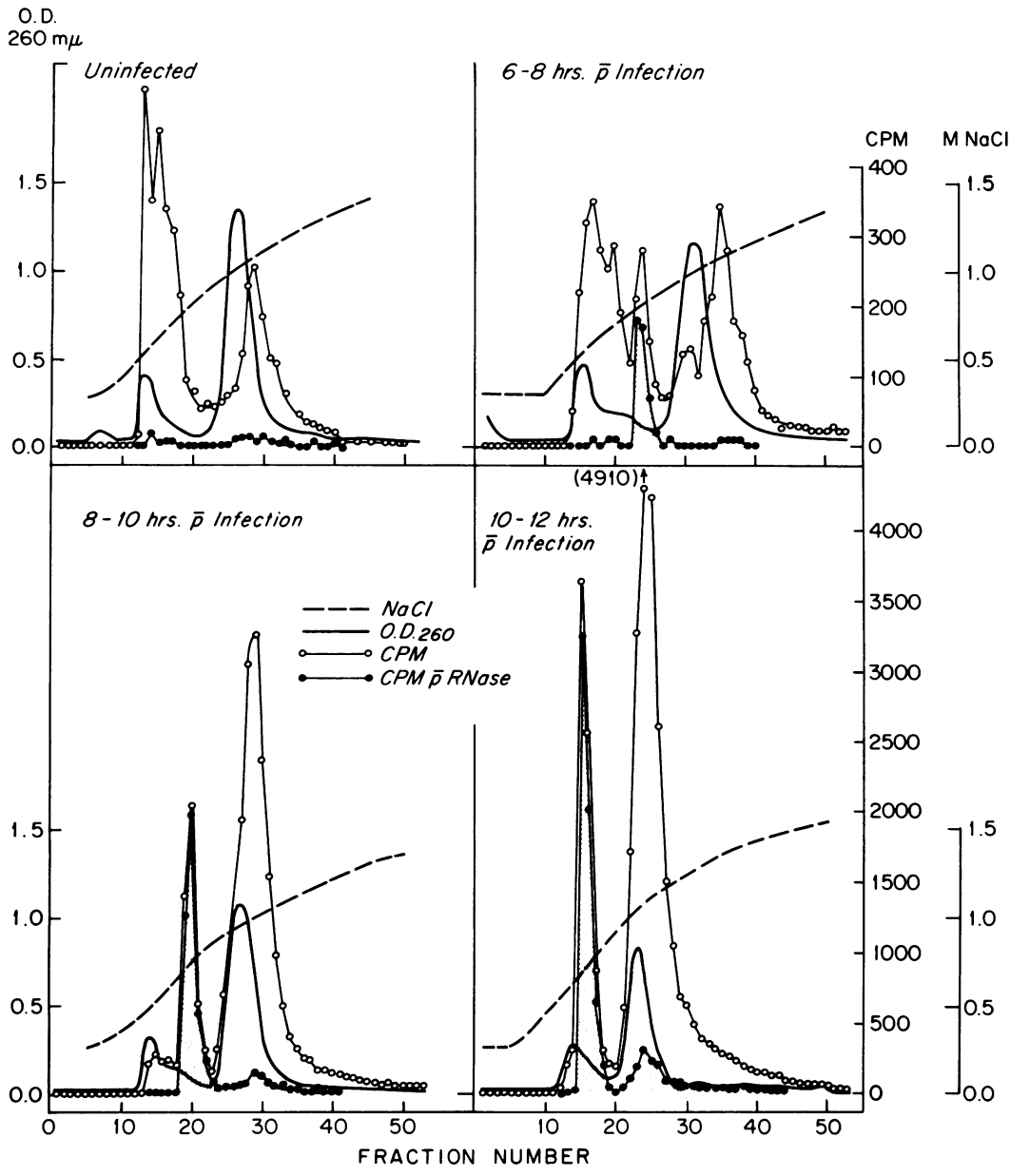

FIG. 4. *Chromatography of RNA on methylated albumin columns. Phenol-extracted RNA was dissolved at a concentration of about 0.2 mg/ml in 0.3 M NaCl and 0.05 M phosphate buffer (pH 6.8) and loaded onto the column. RNA was eluted at 35 C by applying a linear gradient of 0.3 to 1.6 M NaCl in phosphate buffer. Eluted samples were tested for acid-precipitable radioactivity before* (○) *and after* (●) *treatment with ribonuclease.*

which had been extracted from reovirus and further purified by equilibrium density gradient centrifugation in Cs_2SO_4 (33). As shown in Table 1, the double-stranded RNA synthesized 6 to 9 and 9 to 12 hr after infection resembled purified virus RNA with a guanylic plus cytidylic acid (G + C) content of 47.4 to 48.8% and a purine to pyrimidine ratio close to one. The single-stranded RNA also contained about 47% G+C but consistently had a higher proportion of guanylic than cytidylic acid.

Annealing of single-stranded RNA and reo-

TABLE 1. *Base composition analysis of reovirus RNA*

Source of RNA	Cytidylic acid (C)	Adenylic acid (A)	Guanylic acid (G)	Uridylic acid (U)	Per cent G + C	$\frac{A+G}{C+U}$
Purified reovirus	23.5[a]	26.2[a]	24.0[a]	26.3[a]	47.5	1.01
Infected cells						
Double-stranded						
6 to 9 hr postinfection (PI)	24.8	25.0	24.0	26.2	48.8	0.96
9 to 12 hr PI	24.2	26.1	23.2	26.5	47.4	0.97
Single-stranded						
6 to 9 hr PI	22.0	27.3	24.6	26.1	46.6	1.08
9 to 12 hr PI	22.2	25.6	25.2	27.0	47.4	1.03

[a] Figures represent moles per 100 moles of all nucleotides.

TABLE 2. *Annealing of reovirus-directed single-stranded RNA*

Addition to RNA	Annealed	Ribonuclease	Acid-precipitable radioactivity[a]	Per cent ribonuclease-resistant
None	−	−	521	—
None	−	+	34	7
None	+	+	64	12
Denatured reovirus RNA (27 μg)	+	+	451	87
Native reovirus RNA (27 μg)	+	+	45	9
L-cell RNA (16 μg)	+	+	53	10
Denatured L-cell DNA (38 μg)	+	+	26	5

[a] Expressed in counts per minute per 0.6 ml of buffer.

virus RNA. Base sequence homology between single-stranded RNA isolated from infected cells and double-stranded RNA extracted from purified virus was tested by annealing experiments. RNA was purified from cells exposed to uridine-2-C^{14} 10 to 12 hr PI, and the single-stranded RNA was separated on a MAK column. It was annealed with the additions indicated in Table 2. Before heating, more than 90% of the RNA was degraded to acid-soluble material by incubation with ribonuclease. After heating and cooling, the RNA remained nuclease-sensitive. However, when heat-denatured reovirus RNA was present during the heating and cooling, 87% of the single-stranded RNA was converted to a ribonuclease-resistant form. Native reovirus RNA or L-cell RNA and heat-denatured L-cell DNA did not form ribonuclease-resistant complexes with the single-stranded RNA. Single-stranded RNA isolated from uninfected, actinomycin-treated cells and eluted from a MAK column at 1.0 to 1.2 M NaCl also failed to become nuclease-resistant when annealed with denatured reovirus RNA.

Annealing of the single-stranded RNA with denatured reovirus RNA resulted in its conversion to double-stranded RNA (Fig. 5). Single-stranded radioactive RNA was extracted from infected cells and purified by MAK column chromatography. The RNA which eluted at 1.0 to 1.2 M NaCl was collected, annealed with denatured reovirus RNA, and rechromatographed. Its elution shifted to 0.7 to 0.8 M NaCl, the position of double-stranded RNA. The broader elution profile of the annealed RNA as compared to unheated, double-stranded RNA is the consequence of at least two factors which lead to increased heterogeneity. Thermal degradation occurred during both the heat denaturation of the double-stranded RNA and the annealing process, thus resulting in the formation of smaller molecules which elute at a lower salt concentration. A broadening effect in the opposite direction also resulted, because the annealed complexes contain 10 to 20% single-stranded, ribonuclease-sensitive RNA, and because single-stranded RNA elutes at a higher NaCl molarity than double-stranded RNA.

The extent of nuclease resistance attained during annealing was dependent upon the concentration of denatured reovirus RNA. In the experiment shown in Fig. 6, a constant amount (443 counts per min) of radioactive single-stranded RNA, purified from cells exposed to uridine-2-C^{14} 10 to 12 hr PI, was annealed with increasing quantities of denatured reovirus RNA. In the absence of denatured reovirus RNA, 4% of the radioactivity was ribonuclease-resistant. This fraction increased sharply to 50% at a concentration of 4.5 μg of denatured virus RNA per ml and increased more slowly thereafter to 81% at 45 μg/ml. Maximal values of 83 and 85% for nuclease resistance were also obtained in similar experiments with RNA synthesized 6 to 8 and 8 to 10 hr after infection.

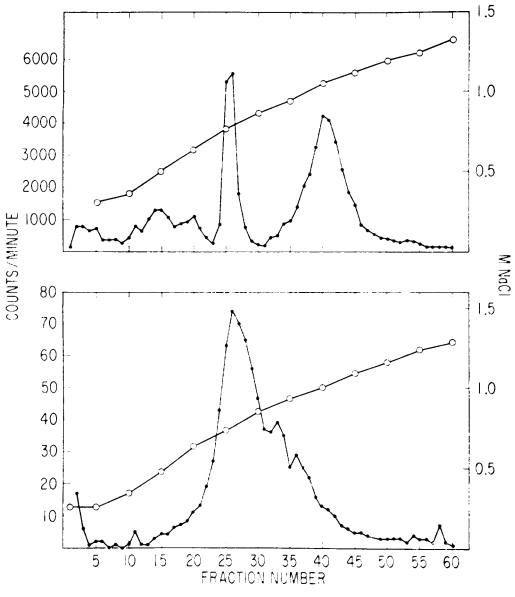

FIG. 5. *Chromatography of reovirus-directed single-stranded RNA before and after annealing with denatured reovirus RNA. RNA was extracted from infected cells incubated with radioactive phosphate-P^{32} 9 to 12 hr postinfection. It was chromatographed on a MAK column (upper panel). Fractions 38 and 39 from the peak of single-stranded RNA were dialyzed against 0.005 M phosphate buffer (pH 7) and 0.3 M NaCl and were annealed with 43 μg/ml of heat-denatured reovirus RNA. The annealed RNA was then rechromatographed (lower panel).*

An additional control experiment with heterologous, denatured double-stranded RNA was performed to test the specificity of the reaction. Single-stranded RNA synthesized 8 to 10 or 10 to 12 hours PI was annealed with heat-denatured rice dwarf virus RNA (Fig. 6). No increase in ribonuclease-resistance above the background level was observed, indicating that there is no detectable base sequence homology between the double-stranded RNA of rice dwarf virus and of reovirus.

It has been demonstrated previously that strand selection occurs when DNA is transcribed by RNA polymerase in vivo (1, 10–12, 20, 37) and may occur under certain conditions in vitro as well (13). The observation that the single-stranded RNA fails to self-anneal (Table 2) suggested that one of the strands of the double-stranded RNA of reovirus is replicated preferentially during infection. Since the annealing process is inefficient at low RNA concentrations (Fig. 6), the lack of self-annealing may also be due to the presence of one or both strands at a concentration inadequate to permit the formation of paired strands. Virus-specific, single-stranded RNA, and ribosomal RNA elute from a MAK column at the same salt molarity (Fig. 4). Consequently, it is not possible to measure the concentration of the single-stranded RNA directly. Double-stranded RNA can be purified free from cell RNA by MAK chromatography, and the concentration required for renaturation after separation of the strands by heating can be measured. If it is assumed that the double-stranded and single-stranded reovirus-directed RNA are synthesized in infected cells from a common precursor pool, the specific activities of the two types of RNA isolated from the same cells will be similar, and the level of radioactivity can be used to approximate the RNA concentration. An infected culture was incubated with uridine-2-C^{14} 10 to 12 hr PI, and the single and double-stranded species were separated on a MAK column. Double-stranded RNA was heat-denatured and then self-annealed at a concentration of 178 counts per min (0.35 μg) in 0.6 ml of buffer. As shown in Table 3, 41% of the radioactivity was ribonuclease-resistant after annealing. On the other hand, the single-stranded RNA at a 20-fold greater radioactivity level did not self-anneal. These results suggest that the failure of single-stranded RNA to self-anneal is not a concentration effect and that one of the strands of helical reovirus RNA is

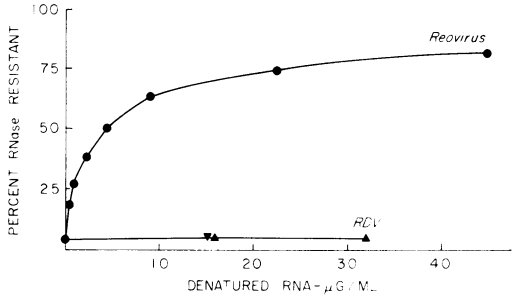

FIG. 6. *Annealing of single-stranded reovirus-directed RNA. Actinomycin-treated cells were incubated with uridine-2-C^{14} 10 to 12 hr postinfection (PI). Newly formed, single-stranded RNA was separated on a MAK column and dialyzed against 0.005 M phosphate buffer (pH 7) and 0.3 M NaCl. To 0.5 ml of this solution (443 counts per min) was added 0.1 ml of 0.1 × SSC containing the indicated quantity of heat-denatured RNA extracted from purified reovirus (●) or rice dwarf virus (▲). The mixture was cooled slowly from 90 C to room temperature, treated with ribonuclease (2 μg/ml, at 37 C for 30 min), precipitated with acid at 4 C, and counted. RNA synthesized in actinomycin-treated cells 8 to 10 hr PI was also purified and annealed with denatured rice dwarf virus RNA (▼).*

TABLE 3. *Annealing of single-stranded and denatured double-stranded RNA*

Addition to RNA	Annealed	Ribonuclease	Single-stranded		Double-stranded	
			Acid-precipitable radioactivity[a]	Per cent ribonuclease-resistant	Acid-precipitable radioactivity[a]	Per cent ribonuclease-resistant
None	−	−	4,042	—	178	—
None	−	+	153	4	5	3
None	+	+	234	6	72	41
Denatured reovirus RNA (27 μg)	+	+	3,351	82	—	—

[a] Expressed in counts per minute per 0.6 ml of buffer.

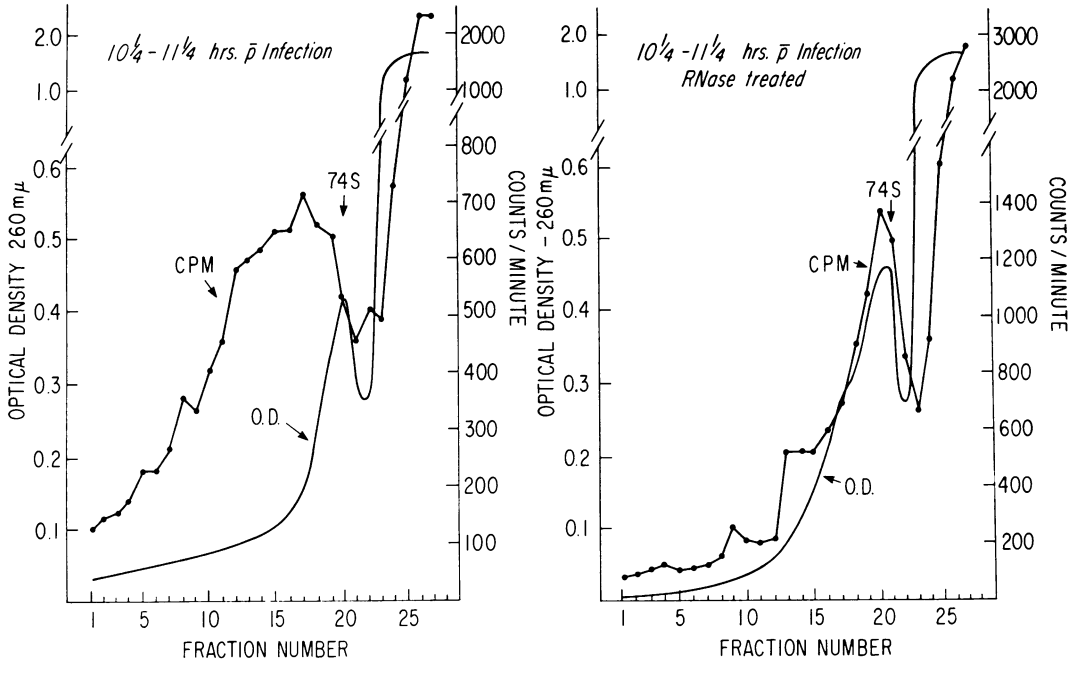

FIG. 7. *Sedimentation of cytoplasmic fractions from reovirus-infected cells. Actinomycin-treated cultures were exposed to uridine-2-C¹⁴ 10.25 to 11.25 hr postinfection. The cells were chilled, suspended in hypotonic media, and ruptured in a glass tissue homogenizer. After removing the unbroken cells and nuclei by low-speed centrifugation, one-half of the extract was made 0.25% with respect to sodium deoxycholate. The second half was incubated for 10 min at 4 C with 2 μg of ribonuclease per ml followed by the addition of detergent. The extracts were then centrifuged through 5 to 30% sucrose density gradients (2.5 hr, at 24,000 rev per min in Spinco SW 25.1 rotor), and the fractions collected were assayed for acid-precipitable radioactivity. The gradients contained 19,480 (left) and 19,960 (right) counts per min of which 2,850 and 1,050 counts per min, respectively, were present in pellets.*

preferentially copied during synthesis of single-stranded, virus-directed RNA.

Polyribosome association of single-stranded RNA. The basic unit of protein synthesis in a number of diverse organisms is the polyribosome, a cluster of single ribosomes held together by a single-stranded messenger RNA (4, 39, 40). Viral protein synthesis also occurs on polyribosomes (28), and, at least in the case of poliovirus replication, the single-stranded viral RNA functions as the messenger RNA (25, 35). In reovirus-infected cells, a fraction of the single-stranded, virus-directed RNA is also present in polyribosomes. Actinomycin-treated cells which had been exposed to uridine-2-C^{14} for 1 hr beginning 10.25 hr PI were homogenized, and the nuclei and unbroken cells were sedimented by low-speed centrifugation. The supernatant

TABLE 4. *Annealing of RNA extracted from polyribosomes*

Addition to RNA	Annealed	Ribonuclease	Acid-precipitable radioactivity[a]	Per cent ribonuclease-resistant
None	−	−	460	—
None	−	+	35	8
None	+	+	61	13
Denatured reovirus RNA (27 μg)	+	+	386	84

[a] Expressed in counts per minute per 0.6 ml of buffer.

fraction which contained about 60% of the total acid-precipitable radioactivity was then centrifuged in a 5 to 30% sucrose density gradient. The acid-precipitable radioactivity was associated with a broad peak in the polyribosome region of the gradient. It sedimented more rapidly than the 74*S* single ribosomes (Fig. 7, left panel). When the extract was digested before centrifugation with 2 μg of ribonuclease per ml for 10 min at 4 C, conditions which partially digest single-stranded RNA, the polyribosomes were degraded to single ribosomes. The partially digested virus-specific RNA remained attached to the single ribosomes and sedimented as a peak of radioactivity at 74*S* (Fig. 7, right panel).

Polyribosome-associated, virus-directed RNA was purified by phenol extraction of fractions 1 to 21 from a gradient identical to that in Fig. 7, left panel. The purified RNA was ribonuclease-sensitive in the presence of 0.25 M NaCl, indicating that it is single-stranded (Table 4). After annealing with denatured reovirus RNA, it was more than 80% ribonuclease-resistant.

Transitory dependence of virus-specific RNA formation on protein synthesis. Purified, double-stranded reovirus RNA does not function in vitro as a template for DNA-dependent RNA polymerase or DNA polymerase of *Escherichia coli* (33). Although the possibility remains that the polymerases of L cells in tissue culture can use intact reovirus RNA as a template, the results of the following experiment indicate that the induction of new enzymes is required for reovirus replication. A series of actinomycin-treated, infected cultures was set up, and at hourly intervals during the infectious cycle protein synthesis was inhibited by adding 2 μg of cycloheximide per ml. This concentration reduced protein synthesis by 90% in less than 1 hour. All cultures were incubated with uridine-2-C^{14} (4×10^{-6} M, specific activity = 24.4 μc/μmole) 11 to 12 hr PI, and the specific activity of the RNA was then determined. As shown in Fig. 8, there was no virus-specific RNA synthesized in cultures inhibited at 6 hr PI or earlier. From the 6th to the 9th hour PI, virus-specific RNA synthesis became progressively resistant to inhibition by cycloheximide.

The effect of cycloheximide on virus-specific RNA synthesis was not due to a direct inhibitory action of the compound on RNA synthesis. In control experiments with growing cells, RNA synthesis was reduced by only 20% after exposure to 2 μg of cycloheximide per ml for a 7-hr period. In addition, the low rate of uridine incorporation into RNA in actinomycin-treated, uninfected cells was not diminished by treatment with cycloheximide for 8 hr (Fig. 8).

Differential inhibition of double-stranded RNA synthesis by cycloheximide late in infection. Total virus-specific RNA synthesis at 11 to 12 hr PI was not detectably reduced by treatment with cycloheximide beginning 9 to 10 hr PI (Fig. 8). However, analysis of the RNA formed under these conditions revealed that there was a highly selective inhibition of the synthesis of double-stranded RNA. An infected, actinomycin-treated culture was divided, and, at 10 hr PI, one of the

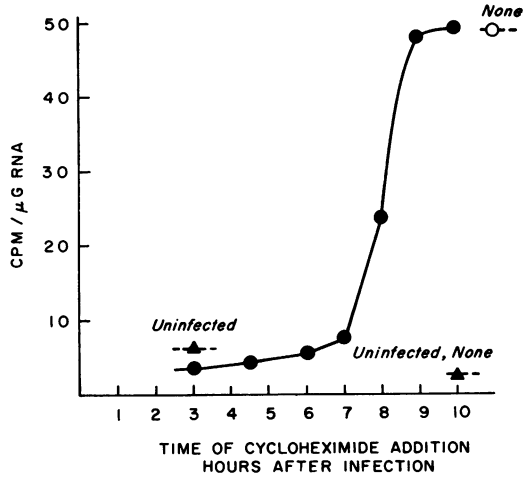

FIG. 8. *Effect of cycloheximide on reovirus-directed RNA synthesis. A series of actinomycin-treated, infected cell cultures was set up and incubated at 37 C. At the indicated times (●), one in the series received 2 μg of cycloheximide per ml to inhibit protein synthesis. All cultures were incubated with uridine-2-C^{14} 11 to 12 hr postinfection. The final specific activity of the newly synthesized RNA in each culture is plotted at the time of cycloheximide addition and is compared to an infected culture receiving no cycloheximide (○). Uninfected cells (▲) were similarly treated but without the addition of virus. Values are shown for a culture which received no cycloheximide and for one which was incubated for 8 hr prior to the addition of uridine-2-C^{14}.*

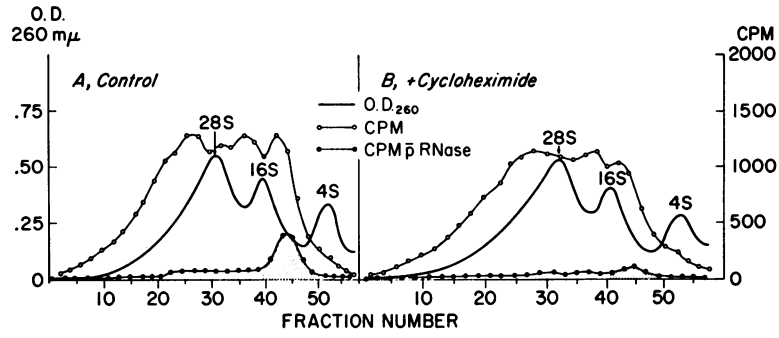

FIG. 9. *Inhibition of double-stranded RNA synthesis by cycloheximide. At 10 hr postinfection (PI), an infected, actinomycin-treated culture was divided and 2 μg of cycloheximide per ml was added to one of the resulting cultures. Both were incubated with uridine-2-C^{14} 11 to 12 hr PI. The RNA was extracted and centrifuged in 15 to 30% linear sucrose density gradients as described in the legend to Fig. 3.*

resulting cultures received 2 μg of cycloheximide per ml. Both were then incubated with uridine-2-C^{14} (specific activity = 24.4 μc/μmole, 4×10^{-6} M) from 11 to 12 hr PI. The RNA was extracted and sedimented in sucrose density gradients. As shown in Fig. 9A, 10% of the total RNA synthesized in the absence of cycloheximide was ribonuclease-resistant. In the culture which had received cycloheximide at 10 hr PI, double-stranded RNA synthesis was reduced by more than 50%, whereas single-stranded RNA synthesis was not inhibited (Fig. 9B).

DISCUSSION

In reovirus-infected cells in which host RNA synthesis has been selectively inhibited with 0.5 μg of actinomycin per ml, synthesis of both single and double-stranded virus-induced RNA can be detected beginning at 6 to 7 hr PI. The double-stranded, nuclease-resistant RNA has a base composition identical to that of RNA extracted from reovirus and purified by isopycnic sedimentation in Cs_2SO_4. Presumably, it is destined for incorporation into mature virus. The guanylic + cytidylic acid content of 47.5%, which differs from the previously reported value of 44% (6), agrees well with the composition expected on the basis of spectrophotometric measurements (Henley, Fresco, and Shatkin, *unpublished data*). Double-stranded RNA that is extracted from infected cells sediments in a sucrose gradient as a homogeneous peak at 10 to 12*S*, the position of RNA from purified reovirus. This corre: ponds to a molecular weight of about 2 million for native DNA (2). The reovirus particle contains an amount of RNA equivalent to 10 million daltons (8), and the double-stranded nucleic acid apparently is degraded during phenol extraction from either purified virus (5, 14; Dunnebacke and Kleinschmidt, *unpublished data*) or infected cells.

The single-stranded, virus-specific RNA is a heterogeneous mixture of molecules which range in size from about 8*S* to more than 40*S* as determined by sucrose gradient analysis. Association of a fraction of the single-stranded RNA with ribosomes to form polyribosomes suggests that one of its functions is to serve as the messenger for virus protein synthesis. When annealed with denatured reovirus RNA, it forms ribonuclease-resistant double-stranded RNA. Thus, the sequence of bases in the single-stranded RNA and virus RNA must be very similar, if not identical; i.e., the single-stranded RNA is coded for by the double-stranded RNA. The finding that the single-stranded RNA does not self-anneal suggests that one strand of the duplex or alternate regions of the two strands are copied selectively. However, this interpretation is subject to the reservation that the specific activities of single-stranded and double-stranded RNA isolated from the same cells are similar, an assumption which may not be valid.

Purified, double-stranded RNA is not a template for nucleic acid polymerases of *E. coli* in vitro (33). On this basis, it might be anticipated that the induction of new enzymes would be required for reovirus replication. In agreement with this possibility, it has been observed that virus-specific RNA synthesis is prevented when protein synthesis is blocked by the addition of cycloheximide at 6 hr PI or earlier in the infectious cycle. The postulated enzymes are apparently formed from 6 to 9 hr PI, since it is during this interval that virus-specific RNA synthesis becomes resistant to cycloheximide inhibition. The synthesis of single-stranded RNA, which comprises the major fraction of the virus-directed RNA, does not diminish for at least 3 hr when cycloheximide is added at 9 hr PI. In contrast, double-stranded RNA synthesis is reduced by more than 50% within 1 hr. These

findings suggest that the two types of RNA are synthesized by different enzymes and that the protein responsible for the formation of double-stranded RNA turns over more rapidly. Kudo and Graham have reported similar findings using puromycin to inhibit protein synthesis (17).

If new enzymes whose synthesis is directed by single-stranded messenger RNA are required for reovirus replication, then it seems possible that the cell genome codes for the enzyme which synthesizes the reovirus-specific single-stranded RNA. The single-stranded RNA could then direct the synthesis of both virus coat proteins and an enzyme for the formation of double-stranded virus RNA. This second enzyme might produce a duplex by replicating a complementary strand on a template of newly formed single-stranded RNA or by a semiconservative mechanism with the parental double-stranded RNA as primer. Alternatively, the parental double-stranded RNA may be reduced to a functional single-stranded messenger RNA inside the cell, or the virus particle, in addition to double-stranded RNA, may contain a small undetected amount of single-stranded RNA adequate to code for a new enzyme. A detailed understanding of the enzymatic mechanisms by which these RNA molecules are synthesized will require a study of purified enzymes from reovirus-infected cells.

ACKNOWLEDGMENTS

We thank K. Miura for the kind gift of rice dwarf virus RNA. R. Ruhl provided excellent technical assistance.

This investigation was supported by the World Health Organization.

LITERATURE CITED

1. BAUTZ, E. K. F. 1963. The structure of T4 messenger RNA in relation to messenger function. Cold Spring Harbor Symp. Quant. Biol. **28**: 205–210.
2. BURGI, E., AND A. D. HERSHEY. 1963. Sedimentation rate as a measure of molecular weight of DNA. Biophys. J. **3**:309–321.
3. EAGLE, H. 1959. Amino acid metabolism in mammalian cell cultures. Science **130**:432–437.
4. GIERER, A. 1963. Function of aggregated reticulocyte ribosomes in protein synthesis. J. Mol. Biol. **6**:148–157.
5. GOMATOS, P. J., AND W. STOECKENIUS. 1964. Electron microscope studies on reovirus RNA. Proc. Natl. Acad. Sci. U.S. **52**:1449–1455.
6. GOMATOS, P. J., AND I. TAMM. 1963. Animal and plant viruses with double-helical RNA. Proc. Natl. Acad. Sci. U.S. **50**:878–885.
7. GOMATOS, P. J., AND I. TAMM. 1963. Macromolecular synthesis in reovirus-infected L cells. Biochim. Biophys. Acta **72**:651–653.
8. GOMATOS, P. J., AND I. TAMM. 1963. The secondary structure of reovirus RNA. Proc. Natl. Acad. Sci. U.S. **49**:707–714.
9. GOMATOS, P. J., I. TAMM, S. DALES, AND R. M. FRANKLIN. 1962. Reovirus type 3: Physical characteristics and interaction with L cells. Virology **17**:441–454.
10. GUILD, W. R., AND M. ROBISON. 1963. Evidence for message reading from a unique strand of pneumococcal DNA. Proc. Natl. Acad. Sci. U.S. **50**:106–112.
11. HALL, B. D., M. GREEN, A. P. NYGAARD, AND J. BOEZI. 1963. The coding of DNA in T2-infected *E. coli*. Cold Spring Harbor Symp. Quant. Biol. **28**:201–204.
12. HAYASHI, M., M. N. HAYASHI, AND S. SPIEGELMAN. 1963. Restriction of *in vivo* genetic transcription to one of the complementary strands of DNA. Proc.Natl. Acad. Sci. U.S. **50**:664–672.
13. HAYASHI, M., M. N. HAYASHI, AND S. SPIEGELMAN. 1963. DNA circularity and the mechanism of strand selection in the generation of genetic messages. Proc. Natl. Acad. Sci. U.S. **51**:351–359.
14. KLEINSCHMIDT, A. K., T. H. DUNNEBACKE, R. S. SPENDLOVE, F. L. SCHAFFER, AND R. F. WHITCOMB. 1964. Electron microscopy of RNA from reovirus and wound tumor virus. J. Mol. Biol. **10**:282–288.
15. KUBINSKI, H., AND G. KOCH. 1966. Regulation of the synthesis of various ribonucleic acids in animal cells. Biochem. Biophys. Res. Commun. **22**:346–351.
16. KUDO, H., AND A. F. GRAHAM. 1965. Synthesis of reovirus ribonucleic acid in L cells. J. Bacteriol. **90**:936–945.
17. KUDO, H., AND A. F. GRAHAM. 1966. Selective inhibition of reovirus-induced RNA in L cells. Biochem. Biophys. Res. Commun. **24**:150–155.
18. LANGRIDGE, R., AND P. J. GOMATOS. 1963. The structure of RNA. Reovirus RNA and transfer RNA have similar three-dimensional structures, which differ from DNA. Science **141**: 694–698.
19. MANDELL, J. D., AND A. D. HERSHEY. 1960. A fractionating column for analysis of nucleic acids. Anal. Biochem. **1**:66–77.
20. MARMUR, J., AND C. M. GREENSPAN. 1963. Transcription *in vivo* of DNA from bacteriophage SP8. Science **142**:387–389.
21. MEJBAUM, W. 1939. Estimation of small amounts of pentose especially in derivatives of adenylic acid. Hoppe-Seylers Z. Physiol. Chem. **258**:117–120.
22. MIURA, K. I., I. KIMURA, AND N. SUZUKI. 1966. Double-stranded ribonucleic acid from rice dwarf virus. Virology **28**:571–579.
23. MIURA, K. I., AND A. MUTO. 1965. Lack of messenger RNA activity of a double-stranded RNA. Biochim. Biophys. Acta **108**:707–709.
24. NIRENBERG, M. W., AND J. H. MATTHAEI. 1961. The dependence of cell-free protein synthesis in *E. coli* upon naturally occurring or synthetic polyribonucleotides. Proc. Natl. Acad. Sci. U.S. **47**:1588–1602.
25. PENMAN, S., K. SCHERRER, Y. BECKER, AND J. E.

DARNELL. 1963. Polyribosomes in normal and poliovirus-infected HeLa cells and their relationship to messenger RNA. Proc Natl. Acad. Sci. U.S. **49**:654–662.

26. REICH, E., R. M. FRANKLIN, A. J. SHATKIN, AND E. L. TATUM. 1962. Action of actinomycin D on animal cells and viruses. Proc. Natl. Acad. Sci. U.S. **48**:1238–1245.
27. SALZMAN, N. P., A. J. SHATKIN, AND E. D. SEBRING. 1964. The synthesis of a DNA-like RNA in the cytoplasm of HeLa cells infected with vaccinia virus. J. Mol. Biol. **8**:405–416.
28. SCHARFF, M. D., A. J. SHATKIN, AND L. LEVINTOW. 1963. Association of newly formed viral protein with specific polyribosomes. Proc. Natl. Acad. Sci. U.S. **50**:686–694.
29. SCHERRER, K., AND J. E. DARNELL. 1962. Sedimentation characteristics of rapidly labelled RNA from HeLa cells. Biochem. Biophys. Res. Commun. **7**:486–490.
30. SEBRING, E. D., AND N. P. SALZMAN. 1964. An improved procedure for measuring the distribution of $P^{32}O_4^{\equiv}$ among the nucleotides of ribonucleic acid. Anal. Biochem. **8**:126–129.
31. SHATKIN, A. J. 1962. Actinomycin inhibition of ribonucleic acid synthesis and poliovirus infection of HeLa cells. Biochim. Biophys. Acta **61**:310–313.
32. SHATKIN, A. J. 1965. Actinomycin and the differential synthesis of reovirus and L cell RNA. Biochem. Biophys. Res. Commun. **19**:506–510.
33. SHATKIN, A. J. 1965. Inactivity of purified reovirus RNA as a template for *E. coli* polymerases *in vitro*. Proc. Natl. Acad. Sci. U.S. **54**:1721–1728.
34. SINGER, M. F., O. W. JONES, AND M. W. NIRENBERG. 1963. The effect of secondary structure on the template activity of polyribonucleotides. Proc. Natl. Acad. Sci. U.S. **49**:392–399.
35. SUMMERS, D. F., AND L. LEVINTOW. 1965. Constitution and function of polyribosomes of poliovirus-infected HeLa cells. Virology **27**:44–53.
36. TAKANAMI, M., AND T. OKAMOTO. 1963. Interaction of ribosomes and polydeoxyribonucleotides. Biochem. Biophys. Res. Commun. **13**: 297–302.
37. TOCCHINI-VALENTINI, G. P., M. STODOLSKY, A. AURISICCHIO, M. SARNAT, F. GRAZIOSI, S. B. WEISS, AND E. P. GEIDUSCHEK. 1963. On the asymmetry of RNA synthesis *in vivo*. Proc. Natl. Acad. Sci. U.S. **50**:935–942.
38. TOMITA, K. I., AND A. RICH. 1964. X-ray diffraction investigations of complementary RNA. Nature **201**:1160–1163.
39. WARNER, J. R., P. M. KNOPF, AND A. RICH. 1963. A multiple ribosomal structure in protein synthesis. Proc. Natl. Acad. Sci. U.S. **49**:122–129.
40. WETTSTEIN, F. O., T. STAEHELIN, AND H. NOLL. 1963. Ribosomal aggregate engaged in protein synthesis: Characterization of the ergosome. Nature **197**:430–435.