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Absence of Adenine-Rich Ribonucleic Acid from Purified Infectious Reovirus 3

ROBERT M. KRUG AND PETER J. GOMATOS
The Sloan-Kettering Institute, New York, New York 10021

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The basic properties of released and cell-associated reovirus are the same. Both contained as their total nucleic acid complement only double-stranded ribonucleic acid (RNA) with an adenine content of 28%. Preparations of purified cell-associated virus, but not released virus, contained adenine-rich RNA which could be separated from the virus with little or no loss of infectivity. These adenine-rich ribonucleic acids were present in the virus preparations either as free RNA or associated with some structures of molecular weight less than 25×10^6 daltons. In contrast to our previous report, double-stranded reovirus RNA possessed little or no template activity for the *Escherichia coli* deoxyribonucleic acid and RNA polymerases.

Reovirus is an icosahedral virus containing double-stranded ribonucleic acid (RNA; references 11 and 19). Gomatos and Tamm (12, 13) reported that the total RNA extracted from purified preparations of reovirus 3 Dearing had an adenine content of 28% and complementary base ratios. This was evidence that the reovirus genome consisted of only double-stranded RNA.

Since then, several reports have appeared describing the presence in purified preparations of cell-associated reovirus of an adenine-rich RNA which was 15 to 20% (1, 2), 25% (24), or 30% (17) of the total nucleic acid of reovirus. It was concluded by these authors that the adenine-rich RNA, as well as double-stranded RNA, was within the reovirus particle.

The present report is a confirmation of the original reports of Gomatos and Tamm (12, 13). The total RNA extracted from purified reovirus 3 Dearing, from cell-associated virus (CAV) or released virus (RV), has an adenine content of 28% and complementary base ratios. As indicated by this base composition analysis, purified infectious reovirus 3 Dearing is free of the adenine-rich RNA synthesized in the infected cell. Double-stranded reovirus RNA possesses little or no template activity for the *Escherichia coli* deoxyribonucleic acid (DNA) and RNA polymerases as was reported by Shatkin (23), and which is in contrast to our previous reports (14, 15, 18).

MATERIALS AND METHODS

Cell culture. L cells, strain 929, a continuous cell line derived from mouse fibroblasts, were grown in suspension in Eagle spinner medium (7) supplemented with 5% fetal bovine serum.

Virus. Dearing strain of reovirus type 3, cloned (11), was used as stock virus after two passages. Infectivity was determined by plaque assay (16). Hemagglutination of bovine erythrocytes with virus was performed as described previously (10).

Virus growth. L929 cells in the logarithmic stage of growth were infected at a multiplicity of 10 to 50 plaque-forming units (PFU) per cell. After an adsorption period of 2 hr at room temperature, Eagle spinner medium, supplemented with 2% fetal bovine serum for RV and 7% fetal bovine serum for CAV, was added. The cells, at a density of 5×10^5 to 8×10^5 cells/ml, were incubated in suspension at 37 C on a roller drum. Reovirus 3 is released to a greater extent when the serum concentration in the growth medium during viral growth is 2% rather than 7%. After 44 to 48 hr, cells and cellular debris were removed from the medium by two successive centrifugations at $27,000 \times g$ for 15 min, and the supernatant fluid was used for purification of released virus. For purification of CAV, the cells were collected 20 to 24 hr after infection by centrifugation at $400 \times g$ for 10 min, and the medium was discarded. For the preparation of ^{32}P-labeled cell-associated reovirus, carrier-free ^{32}P-labeled orthophosphate was added to a final concentration of 25 μc/ml in the viral growth medium.

Purification of RV. The supernatant fluid from the $27,000 \times g$ centrifugation of the infected cell suspension was concentrated 20-fold by negative pressure dialysis. The virus in this concentrate was sedimented by centrifugation at $78,000 \times g$ for 3 hr, and the pellet was suspended in 10 ml of phosphate-buffered saline (PBS; reference 6) by means of Dounce homogenization. The resuspended pellet was treated with deoxyribonuclease (30 μg/ml) and pancreatic ribonuclease (2 μg/ml) for 20 min at 37 C, and the suspension was cooled to 0 C. Chymotrypsin (30 μg/ml) was added, and the suspension was left at 0 C for 45 min. To one volume of the enzyme-treated

suspension was added an 0.5 volume of the fluorocarbon, trifluorotrichloroethane (Genesolv-D, Allied Chemical Co., Morristown, N.J.), and the mixture was homogenized for 3 min in an Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.). The aqueous phase was separated from the fluorocarbon phase by centrifugation at 35,000 × *g* for 5 min and was treated a second time with an 0.5 volume of the fluorocarbon for 1 min. The two fluorocarbon layers were retreated with an equal volume of PBS five times. To the combined aqueous layers at 0 C was added solid ammonium sulfate to 60% saturation. The virus was collected by centrifugation at 35,000 × *g* for 20 min. The pellet was taken up in PBS, and solid CsCl was added to achieve an average density of 1.38 g/ml. The mixture was centrifuged at 4 C in an SW50 rotor at 33,000 rev/min for 12 hr, and at 39,000 rev/min for an additional 6 hr. The virus band was collected, and CsCl was removed by chromatography through Sephadex G-50 in PBS.

Purification of CAV. The infected cells were suspended in RSB [0.01 M tris(hydroxymethyl)aminomethane (Tris), *p*H 7.4, 0.01 M KCl, 0.0015 M $MgCl_2$] and ruptured with 25 strokes in a tight-fitting Dounce homogenizer. The resulting homogenate was centrifuged at 250 × *g* for 8 min, and the supernatant fluid, cytoplasmic extract 1, was collected. The nuclei and cellular debris were treated with a mixture of 0.88% Tween 40 and 0.44% deoxycholate in RSB. As described by Penman (22), this treatment leaves nuclei intact. After centrifugation at 250 × *g* for 8 min, the supernatant fluid, cytoplasmic extract 2, was collected. The two cytoplasmic extracts were pooled and made isotonic by the addition of 2 M KCl, and the virus was sedimented by centrifugation at 35,000 × *g* for 20 min (9). The pellet was taken up in PBS, and virus was purified as described above for released virus.

Extraction of reovirus RNA from purified virus. The nucleic acid of the virus was extracted with phenol at room temperature, and the phenol was removed from the aqueous phase by five ether extractions (8). Residual ether was removed under reduced pressure. For determination of base composition, before phenol extraction the purified virus preparation was desalted by chromatography through Sephadex G-50 medium gel with water as the eluant. RNA was extracted as described above, and the resulting aqueous phase was lyophilized to dryness.

Determination of base composition. (i) RNA associated with purified RV was hydrolyzed in 1 N HCl for 1 hr at 100 C and analyzed for base composition by the procedure of Smith and Markham (25).

(ii) ^{32}P-labeled RNA associated with purified CAV was hydrolyzed in 0.2 ml of 0.3 M KOH for 18 hr at 37 C. Potassium ions were removed by descending chromatography on carboxymethyl cellulose paper (Whatman CM-82), and base composition was determined by electrophoresis in 20% acetic acid (*p*H 3.0), as described by Gomatos (9).

(iii) To determine the base composition of ^{32}P-labeled RNA fractionated by gel filtration chromatography on Sephadex G-75, the fractions containing radioactive material were pooled and made 0.3 M in sodium acetate and 0.0001 M in sodium versenate. The RNA was precipitated by the addition of two volumes of ethyl alcohol. After 2 hr at −40 C, the precipitate was collected by centrifugation and dissolved in $\frac{1}{50}$ × KKC (1 × KKC = 0.15 M potassium chloride, 0.015 M potassium citrate); salt was removed by gel filtration chromatography on Sephadex G-10 in water. The RNA was hydrolyzed, and its base composition was determined as described above.

E. coli RNA polymerase. The *E. coli* DNA-dependent RNA polymerase was prepared according to Chamberlin and Berg (4). Assay conditions were those described previously (14), except that spermidine was not included in the reaction mixture.

Analytical procedures. Acid-insoluble radioactivity was determined by precipitating samples with 5% trichloroacetic acid after the addition of 50 to 100 μg of bovine plasma albumin as coprecipitant. The resulting precipitates were collected on HA filters (Millipore Corp., Bedford, Mass.; 0.45 μm pore size), and the amount of radioactivity was determined in a Tri-Carb scintillation spectrometer.

DNA was determined by the diphenylamine reaction as modified by Burton (3), with salmon sperm DNA used as standard. Protein determinations were performed by the method of Lowry et al. (21) using crystalline bovine plasma albumin as standard.

Materials. Carrier-free ^{32}P-labeled orthophosphate, adenosine triphosphate-8-^{14}C and ^{3}H-adenosine triphosphate were obtained from New England Nuclear Corp., Boston, Mass. Unlabeled ribonucleoside triphosphates were obtained from P-L Biochemicals, Milwaukee, Wis. Salmon sperm DNA was obtained from Calbiochem, Los Angeles, Calif. The following were obtained from Worthington Biochemical Corp., Freehold, N.J.: deoxyribonuclease, 1× crystallized; pancreatic ribonuclease, 5× crystallized; and α-chymotrypsin, 3× crystallized. Mid-log-phase *E. coli* B was obtained from General Biochemicals, Chagrin Falls, Ohio.

RESULTS

Purification of reovirus. After 44 to 48 hr, 50 to 60% of the total reovirus produced in L929 mouse fibroblasts was released into the medium without concomitant rupture of the cells as determined by the impermeability of the plasma membrane of the cells to 0.1% trypan blue. Because of the low level of cellular components, RV could be readily purified free of detectable host cell contaminants. In contrast, CAV collected at 20 to 24 hr after infection must be purified from all constituents of the infected cell cytoplasm. Either CAV or RV, not both, was purified from a single batch of infected cells. Table 1 shows the purification schemes.

CAV was collected from the cytoplasmic extract by centrifugation at 35,000 × *g* for 20 min (9). RV could be quantitatively recovered from the medium of the infected cell suspension by centrifugation at 78,000 × *g* for 3 hr. Subse-

TABLE 1. *Purification of reovirus 3*

Steps in purification	Cell-associated virus		Released virus	
	Total PFU	PFU/HAU	Total PFU	PFU/HAU
Cytoplasmic extract 1	212×10^{10}	4.60×10^{6}	NA[a]	NA
Cytoplasmic extract 2	340×10^{10}	—[b]	NA	NA
35,000 × *g* pellet	175×10^{10}	0.90×10^{6}	NA	NA
78,000 × *g* pellet	NA	NA	92.0×10^{10}	1.44×10^{6}
Treatment with DNase and RNase[c]	201×10^{10}	0.52×10^{6}	54.9×10^{10}	0.82×10^{6}
Treatment with chymotrypsin	361×10^{10}	0.41×10^{6}	51.6×10^{10}	0.75×10^{6}
Homogenization with fluorocarbon	300×10^{10}	1.80×10^{6}	35.6×10^{10}	1.29×10^{6}
Ammonium sulfate precipitation	293×10^{10}	2.04×10^{6}	50.3×10^{10}	2.86×10^{6}

[a] Does not apply.
[b] The Tween 40 and deoxycholate in cytoplasmic extract 2 caused hemolysis of the bovine erythrocytes.
[c] DNase, deoxyribonuclease; RNase, ribonuclease.

quently, RV and CAV were purified similarly. Of the infectious virus originally present in the medium or in the cytoplasmic extract, 50 to 100% was recovered after the ammonium sulfate precipitation step (Table 1). This purification is similar to that previously described (8, 11), with a minor modification. In the fluorocarbon homogenization step, the fluorocarbon and aqueous layers were separated by centrifugation at 35,000 × *g* rather than at 1,000 × *g*; the fluorocarbon layer was retreated with PBS five times rather than once, and the pooled aqueous phases were concentrated by ammonium sulfate precipitation. As a result of this modification, resulting in a more effective separation of the aqueous and fluorocarbon layers, the recovery of virus after the fluorocarbon treatment was increased from 10 to 20% to 70 to 100%. As was reported for the previous purification (8, 11), the ratio of PFU to hemagglutinating units (HAU) of virus did not vary significantly in the final purified virus preparation from that present initially (Table 1).

After ammonium sulfate precipitation, the virus was subjected to equilibrium density gradient centrifugation in CsCl. The typical distribution of 10^{11} to 2×10^{11} PFU of either RVor CAV is shown in Fig. 1. The virus band was separated into five fractions, A through E, for analysis. Virus was concentrated at a density of 1.38 g/ml (fraction C in Fig. 1, Table 2) as described previously (11) and spread both above, to a density of 1.37 g/ml (fraction E), and below, to a density of 1.39 g/ml (fraction A). In some preparations, material which banded at a density of 1.34 g/ml was present but was not further studied. When present, membranous material remained at the top of the gradient. It appears (Fig. 1) as if there was a large amount of material at the top of the gradient. In actual fact this was a thin membrane which had partially dislodged from the top and lay at an angle, thus scattering more light and appearing falsely as a heavy band.

After the equilibrium CsCl centrifugation, viral fractions A through E were chromatographed

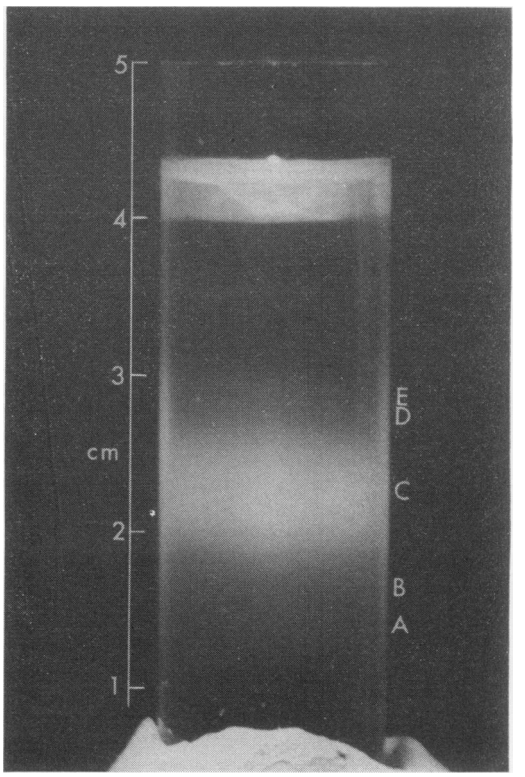

FIG. 1. *Distribution of 1.6 × 10^{11} PFU of reovirus, RV or CAV, after equilibrium density gradient centrifugation in CsCl.*

TABLE 2. *Specific infectivity of fractions from the virus band in cesium chloride*

Virus fraction from CsCl[a]	Released virus		Cell-associated virus	
	Total PFU	PFU/mg of protein	Total PFU	PFU/mg of protein
A	10.9×10^{10}	0.9×10^{10}	7.2×10^{10}	9.1×10^{10}
B	14.5×10^{10}	5.3×10^{10}	7.3×10^{10}	9.0×10^{10}
C	60.8×10^{10}	6.6×10^{10}	58.2×10^{10}	16.7×10^{10}
D	16.1×10^{10}	2.7×10^{10}	5.4×10^{10}	6.6×10^{10}
E	8.1×10^{10}	1.6×10^{10}	3.0×10^{10}	3.9×10^{10}

[a] Defined in Fig. 1.

through Sephadex G-50 medium in PBS before virus was assayed. There was quantitative recovery from fractions A through E of all the PFU present in the ammonium sulfate precipitate. Virus of the highest specific infectivity (PFU per milligram of protein) was in the most concentrated part of the band (fraction C, Table 2). Based on the chemical composition and molecular weight of reovirus (11), this specific infectivity indicates a ratio of approximately 65 particles per PFU.

Reovirus possesses two distinct protein coats (5, 20). It has been suggested that the outer of these two protein coats was dispensable for infectivity since, in some strains of reovirus, treatment with chymotrypsin at 37 C resulted in enhancement of infectivity, attributed to removal of the outer protein coat (26). That the outer protein coat was not removed during the virus purification presented in this paper was indicated by the electron micrographs of reovirus 3 purified similarly (Fig. 1 of reference 5), in which both protein coats were seen morphologically intact in the purified virion, and the virus had a diameter of 75 nm. Furthermore, the purified virus studied in this paper had a density of 1.38 in cesium chloride, the density of morphologically intact reovirus 3 (11), whether or not treatment with chymotrypsin at 0 C was employed during purification. Had the outer protein coat of reovirus been removed partially or totally, the density of the resulting subviral particle would have been higher than that of virus, namely, 1.40, as reported by Gomatos (9), or 1.46, as reported by Loh and Shatkin (20). Thus, the purified virus studied in this paper possessed both protein coats of reovirus.

Base composition of the total RNA of reovirus. To determine the base composition of viral RNA, it was necessary to remove salt from the virus preparation. CsCl-banded RV, after Sephadex G-50 chromatography in PBS, was rechromatographed through Sephadex G-50 medium gel using water as the eluant. The virus was stable to this chromatography: in several experiments, 50 to 90% of the PFU applied to the column was recovered. The virus was totally excluded from the gel, eluting in fractions 4 through 8 (Fig. 2). In the released virus preparation, all the material which absorbed in the ultraviolet range was coincident with the virus peak in the exclusion volume and none was retarded by the gel.

The RNA extracted from different purified preparations of RV [density 1.38 g/ml in CsCl (Fig. 1, fraction C)] had an adenine content of 28% and complementary base ratios (Table 3), confirming the original report of Gomatos and Tamm (12) for released reovirus.

In contrast to RV, gel filtration chromatog-

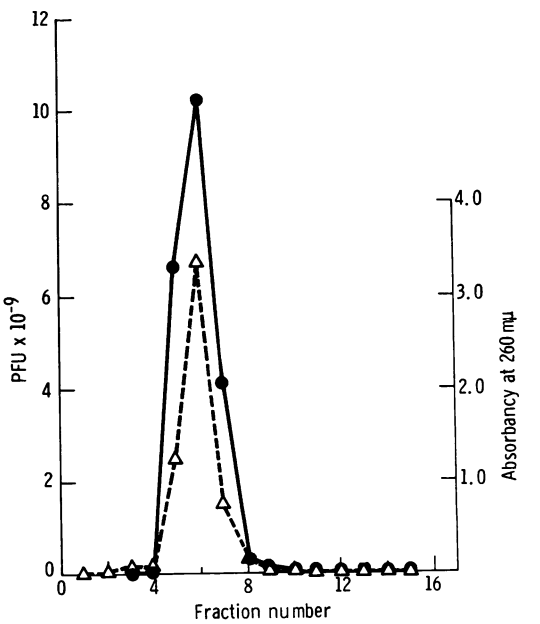

FIG. 2. *Chromatography of purified RV through a column (12 by 1.2 cm) of Sephadex G-50 medium. Water was the eluant and 1.0-ml fractions were collected. Absorbancy at 260 nm (△); PFU (●).*

TABLE 3. *Base composition of the total RNA from RV*

Prepn	Nucleotide (mole fraction)[a]				
	CMP	AMP	GMP	UMP	A + G/ C + U
Reference 12[b]	0.223	0.280	0.220	0.279	1.00
1	0.226	0.284	0.199	0.291	0.94
2	0.216	0.286	0.214	0.284	1.00

[a] Abbreviations: CMP, cytidine monophosphate; AMP, adenosine monophosphate; GMP, guanosine monophosphate; UMP, uridine monophosphate; A, adenine; G, guanine; C, cytosine, U, uracil.

[b] Gomatos and Tamm, average of five determinations.

raphy of ^{32}P-labeled CAV on Sephadex G-50 medium gel in water resulted in the separation from virus of some material labeled with ^{32}P (Fig. 3). The virus was excluded from the gel and eluted in tubes 4 through 8. The respective base compositions of the RNA species extracted with phenol from the virus and from the material in tubes 9 through 11 were then determined.

The RNA which was separated from all preparations of purified CAV during Sephadex G-50 chromatography in water (namely that in tubes 9 through 11, Fig. 3) was adenine-rich (Table 4). The adenine content of the RNA extracted from virus fraction C (defined in Fig. 1) of CAV preparations purified at different times varied from 33.2 to 27.8%. Moreover, within a given purified CAV preparation, the adenine content in the virus fractions A through E varied, with fractions at higher density having higher adenine contents (Table 4; Table 5, preparation I). In some CAV preparations (e.g., Table 5, preparation II), not only the RNA in virus at density 1.38 g/ml (fraction C) but even that in virus at greater densities (fractions A and B) had an adenine content of 28% and complementary base ratios similar to the results for released virus. These results indicated that variable amounts of adenine-rich RNA remained with CAV during the virus purification, and that the purification of CAV could yield infectious virus containing little or no detectable adenine-rich RNA.

Chromatography on Sephadex G-50, which retains material of less than 30,000 daltons, was effective in removing either some or all of the adenine-rich RNA from CAV. This suggested that gel filtration chromatography, utilizing a gel such as Sepharose 2B to exclude reovirus (molecular

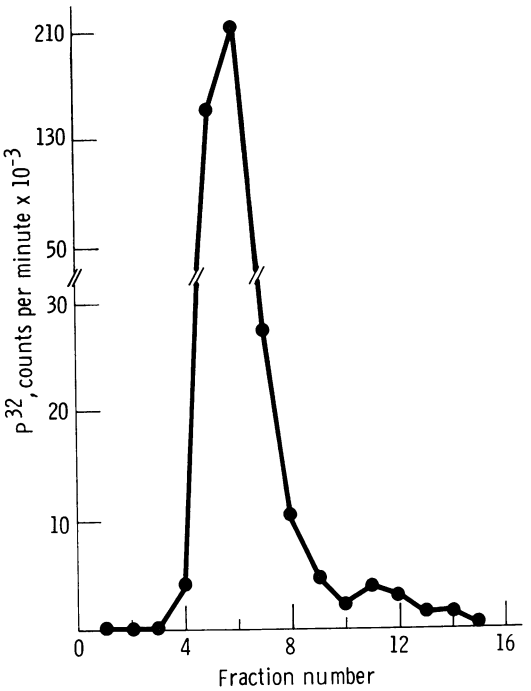

FIG. 3. *Chromatography of purified ^{32}P-labeled CAV through a column (12 by 1.2 cm) of Sephadex G-50 medium. Water was the eluant.*

TABLE 4. *Effect of Sephadex G-50 chromatography on the base composition of ^{32}P-labeled CAV*

Virus fraction from CsCl[a]	RNA eluting with virus[b]				RNA separated from virus			
	CMP	AMP	GMP	UMP	CMP	AMP	GMP	UMP
A	0.199	0.325	0.223	0.252	0.115	0.508	0.091	0.287
B	0.215	0.296	0.223	0.266	0.131	0.489	0.162	0.218
C	0.208	0.318	0.213	0.261	0.088	0.499	0.115	0.297
D	0.222	0.279	0.225	0.274	0.118	0.508	0.120	0.254
E	0.231	0.265	0.233	0.270	0.197	0.304	0.224	0.275

[a] Defined in Fig. 1.

[b] Abbreviations as in footnote *a*, Table 3.

TABLE 5. *Base composition of the total RNA in fractions from the cell-associated virus band in cesium chloride*

Virus fraction from CsCl[a]	Nucleotide (mole fraction)			
	CMP	AMP	GMP	UMP
CAV prepn I				
A	0.175	0.370	0.189	0.267
B	0.180	0.363	0.201	0.256
C	0.193	0.332	0.193	0.281
D	0.196	0.335	0.185	0.283
E	0.205	0.318	0.201	0.275
CAV prepn II				
A	0.233	0.282	0.227	0.259
B	0.224	0.275	0.229	0.273
C	0.233	0.278	0.220	0.269

[a] Defined in Fig. 1.

weight of 70 $\times$ 10^6 daltons) from material of less than 25 $\times$ 10^6 daltons, could separate totally the adenine-rich RNA from all CAV preparations.

A virus fraction C which had an adenine content of 33.2% when determined after Sephadex G-50 chromatography was applied to a Sepharose 2B column (Fig. 4); 63% of the virus was recovered. Of the recovered virus, 93% was excluded from the gel, eluting in fractions 14 through 19. The RNA in the virus in fractions 14 to 16 was free of detectable adenine-rich RNA, and had a base composition, close to complementarity: C, 0.218; A, 0.270; G, 0.219; U, 0.293. The RNA in the virus in fractions 17 to 19 had the same base composition. Of the ^{32}P-labeled material applied to the column, 30% eluted after fraction 19, but the fractions containing adenine-rich RNA were not identified. Thus, it has been possible to free all CAV preparations from adenine-rich RNA without significant loss of infectivity.

Lack of template activity of reovirus RNA with the E. coli DNA and RNA polymerases. With the preparations of RV and CAV containing only double-stranded RNA, we reinvestigated the effectiveness of reovirus RNA as template for the *E. coli* DNA and RNA polymerases. We reported previously that reovirus RNA from RV was a template for the DNA polymerase for the synthesis of a complementary DNA, and was a template for the RNA polymerase for the synthesis of complementary RNA and of the homopolymers, polyadenylate and polyuridylate (14, 15, 18). Subsequent to our previous reports, it was found that reovirus RNA extracted from all RV preparations, purified as described in this report, possessed no template activity with *E. coli* DNA and RNA polymerases for the synthesis of DNA and RNA products similar in base sequence to reovirus RNA. In addition, when CAV was purified from the cytoplasmic extract as described in this report, the RNA from all CAV preparations did not possess template activity for DNA synthesis nor complementary RNA synthesis with the *E. coli* DNA and RNA enzymes.

These results indicated that the template activity previously reported for complementary RNA and DNA synthesis observed with reovirus RNA was probably due to contaminating host cell DNA, as suggested by Shatkin (23). This is the most probable conclusion, despite the fact that, when levels of reovirus RNA saturating for both *E. coli* polymerases, namely 30 μg, were tested at that time in the diphenylamine reaction by which 0.5 μg of DNA can be detected, there was no DNA detectable.

All RV and CAV preparations, purified as described in this report, which possessed an adenine

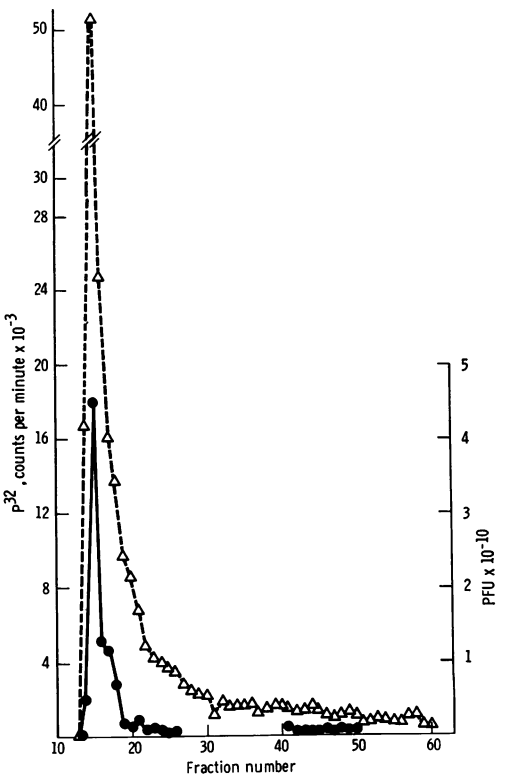

FIG. 4. *Chromatography of purified ^{32}P-labeled CAV through a column (80 $\times$ 1.5 cm) of Sepharose 2B. PBS was the eluant and 2.5-ml fractions were collected. ^{32}P acid-insoluble radioactivity (△); PFU (●).*

content of 28% and complementary base ratios, yielded RNA which had little or no template activity for homopolymer synthesis with the *E. coli* RNA polymerase (Table 6). RNA extracted with phenol from CAV preparations with adenine content greater than 28% (e.g., Table 5, preparation I) also was ineffective as template for the homopolymer syntheses. The latter CAV preparations contained, in addition to double-stranded RNA, adenine-rich ribonucleic acids which were less than 2,000 daltons in molecular weight as determined by gel filtration chromatography on Sephadex G-75.

Since adenine-rich molecules of molecular weight greater than 2,000 had already been described (1, 17, 24), it seemed possible that the adenine-rich molecules described in the present paper were in fact derived from the hydrolysis of larger molecules, and that these larger molecules were the templates for the homopolymer syntheses with the *E. coli* RNA polymerase observed previously (14, 18).

When the temperature of incubation of virus with pancreatic deoxyribonuclease and ribonuclease during purification was lowered from 37 to 24 C, adenine-rich RNA species of greater than 2,000 molecular weight were found. The RNA extracted from such CAV preparations had one of two different distributions after gel filtration chromatography on Sephadex G-75 (Fig. 5). More than 90% of the RNA in the preparations depicted in Fig. 5 eluted in the void volume, had complementary base ratios, and was double-stranded RNA. The preparation shown in Fig. 5A had RNA molecules of 17,000 daltons (effluent volume, 70 ml) and adenine content of 77%; it also had heterogeneous RNA with an adenine content of 55% eluting after the adenine-rich RNA of 17,000 daltons. In the preparation shown in Fig. 5B, only heterogeneous RNA species of molecular weights of 3,000 to 8,000 and adenine content of 50% were present. Template activity for the synthesis of polyuridylate (not shown in Fig. 5) and of polyadenylate with the *E. coli* RNA polymerase was associated with both species of adenine-rich RNA, that of 17,000 daltons and those of lower molecular weight (Fig. 5). Adenine-rich RNA of molecular weight of lower than 2,000 was, as stated above, ineffective as template.

TABLE 6. *Lack of template activity of reovirus RNA for homopolymer synthesis with the E. coli RNA polymerase*[a]

Template	AMP incorporated in 2 hr
	nmole
None	0.038
RNA from RV	0.052
RNA from RV	0.108
RNA from RV	0.119
Salmon sperm DNA	2.140
None	0.105
RNA from CAV	0.155
RNA from CAV	0.197
Salmon sperm DNA	2.230

Different preparations of RV or CAV, adenine content 28%, were extracted with phenol. Ten to 15 μg of the resulting RNA or 22 μg of salmon sperm DNA was used as template for adenosine monophosphate incorporation into RNA in the presence of four nucleoside triphosphates with the *E. coli* RNA polymerase as described. The specific activity of the RNA polymerase was 750 units/mg of protein, using the units defined by Chamberlin and Berg (4).

Thus, it is clear that adenine-rich RNA species present in CAV preparations were responsible for homopolymer synthesis with the *E. coli* RNA polymerase, and that they were derived as breakdown products from either an adenine-rich RNA of 17,000 daltons or from a larger molecule. Adenine-rich RNA was hydrolyzed to molecules of less than 2,000 daltons when the partially purified virus was exposed to 37 C for 20 min; this breakdown was independent of the presence of pancreatic deoxyribonuclease and ribonuclease. Only partial breakdown of adenine-rich RNA occurred when the temperature of incubation was lowered to 24 C. As shown in this paper, adenine-rich RNA species are not necessary for the infectivity of reovirus.

DISCUSSION

The basic properties of released and cell-associated reovirus are the same. Both have a PFU to HAU ratio of 10^6 to 3×10^6, a particle to PFU ratio of approximately 65, and both contain as their total nucleic acid complement only double-stranded RNA, with an adenine content of 28% and complementary base ratios.

A component of the infected cell cytoplasm from which CAV, but not RV, had to be carefully separated was single-stranded adenine-rich RNA. Such adenine-rich RNA was synthesized in reovirus-infected cells in the presence of actinomycin D (1, 2, 24). As shown in the present study, when actinomycin D was not used, the synthesis of adenine-rich RNA was not dependent on the presence of this inhibitor. An RNA species about 60 nucleotides in length and with an adenine content of 88% has been shown to be synthesized at approximately the same time as double-stranded viral RNA (1, 2). This is similar in size and base composition to an adenine-rich RNA

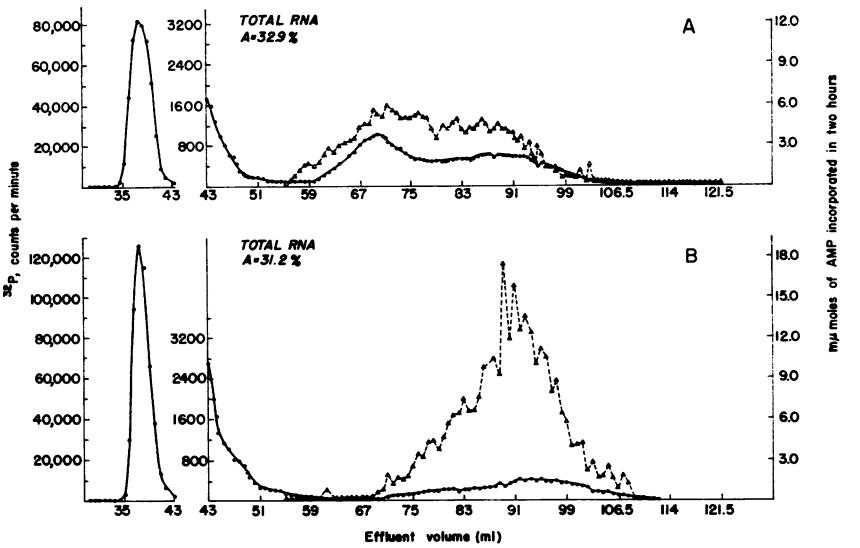

FIG. 5. *Analysis of ^{32}P-labeled RNA from two different cell-associated virus preparations by gel filtration chromatography through Sephadex G-75. The RNA from each of the two virus preparations, containing 140 μg of RNA and 520,000 ^{32}P counts per min, was applied to separate columns (80 by 1.5 cm) of Sephadex G-75 in 0.02 M ammonium acetate, and 0.8-ml fractions were collected. Acid-insoluble radioactivity in each fraction was determined as described. A sample of each fraction was assayed for its capacity to direct adenosine monophosphate incorporation into RNA in the presence of the four nucleoside triphosphates with the E. coli RNA polymerase as described. The specific activity of the RNA polymerase was 1,000 units/mg of protein. Molecular weights were assigned on the basis of calibration of the column with cytochrome c and the standard curve published by Pharmacia Fine Chemicals, Inc. Blue dextran 2000 (molecular weight, 2 × 10^6) eluted in the exclusion volume of the column at 36 ml and cytochrome c (molecular weight, 12,400) eluted from 76 to 80 ml. P^{32} acid-insoluble radioactivity* (●); *adenosine monophosphate incorporated* (△).

detected in the present study. Two other species of adenine-rich RNA molecules were detected with molecular weights of (i) 3,000 to 8,000 daltons and (ii) less than 2,000 daltons. These smaller molecules were derived from breakdown of the larger adenine-rich RNA of 17,000 daltons.

Adenine-rich RNA has been found in purified preparations of reovirus and reported to be within the virus particle (1, 2, 17, 24). This adenine-rich RNA caused the base composition of the total nucleic acid extracted from CAV to deviate from complementary values. However, the present work has clearly shown that adenine-rich RNA is not a necessary component of infectious reovirus 3. Both RV and CAV contain only double-stranded RNA with an adenine content of 28% and complementary base ratios.

The adenine-rich RNA was present in purified CAV preparations in two states: (i) as free RNA, which could be separated from the virus by chromatography through Sephadex G-50; and (ii) at times, in some virus fractions, as RNA associated with a larger structure, which could be separated from the virus by chromatography through Sepharose 2B. The structures with which the adenine-rich RNA was associated had a density which was close to that of virus in CsCl but, as shown in this paper, were not virus. It was probably in this latter protected state that adenine-rich RNA was studied previously, since it was resistant to hydrolysis by micrococcal nuclease, whereas free adenine-rich RNA was sensitive to this enzyme (1, 24). All of the free adenine-rich RNA in our purified CAV preparations was probably derived from that associated with particles, since, in this study, any free adenine-rich RNA present initially would have been removed during purification by the steps requiring precipitation or centrifugation. That no adenine-rich RNA existed free within the infected cell was indicated by the experiments of Bellamy and Joklik (1) which showed that all the adenine-rich RNA present in cytoplasmic extracts from infected cells was sedimented under relatively low centrifugal fields.

The function of adenine-rich RNA in reovirus infection is not known. Adenine-rich RNA species have not been detected in the cytoplasm of uninfected L929 mouse fibroblasts (1; Krug and Gomatos, *unpublished data*). In studies to determine whether the adenine-rich RNA could serve to link the various segments of the reovirus

double-stranded genome, an excess of double-stranded reovirus RNA was added to adenine-rich RNA of high specific activity (1, 24). The mixture was exposed to conditions optimal for annealing and was analyzed by polyacrylamide gel electrophoresis or by chromatography. There was no increase in length of the reovirus RNA components (1). More specifically, however, none of the adenine-rich RNA hybridized with the reovirus RNA (1, 24). This suggests to us that the adenine-rich RNA species are not synthesized on the viral RNA as template, and that long sequences of uridylic acid residues do not exist in reovirus RNA.

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LITERATURE CITED

1. Bellamy, A. R., and W. K. Joklik. 1967. Studies on the A-rich RNA of reovirus. Proc. Nat. Acad. Sci. U.S.A. **58**:1389–1395.
2. Bellamy, A. R., L. Shapiro, J. T. August, and W. K. Joklik. 1967. Studies on reovirus RNA, I. Characterization of reovirus genome RNA. J. Mol. Biol. **29**:1–17.
3. Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315–323.
4. Chamberlin, M., and P. Berg. 1962. Deoxyribonucleic acid-directed synthesis of ribonucleic acid by an enzyme from *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **48**:81–94.
5. Dales, S., P. J. Gomatos, and K. C. Hsu. 1965. Uptake and development of reovirus in strain L cells followed with labeled viral ribonucleic acid and ferritin-antibody conjugates. Virology **25**:193–211.
6. Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exp. Med. **99**:167–182.
7. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. Science **130**:432–437.
8. Gomatos, P. J. 1966. Purification of reovirus 3, Dearing strain and extraction of its RNA, p. 493–497. *In* G. L. Cantoni and D. R. Davies (ed.), Procedures in nucleic acid research. Harper and Row, New York.
9. Gomatos, P. J. 1967. RNA synthesis in reovirus-infected L929 mouse fibroblasts. Proc. Nat. Acad. Sci. U.S.A. **58**:1798–1805.
10. Gomatos, P. J., and I. Tamm. 1962. Reactive sites of reovirus type 3 and their interaction with receptor substances. Virology **17**:455–461.
11. Gomatos, P. J., and I. Tamm. 1963. The secondary structure of reovirus RNA. Proc. Nat. Acad. Sci. U.S.A. **49**:707–714.
12. Gomatos, P. J., and I. Tamm. 1963. Animal and plant viruses with double-helical RNA. Proc. Nat. Acad. Sci. U.S.A. **50**:878–885.
13. Gomatos, P. J., and I. Tamm. 1963. Base composition of the RNA of a reovirus variant. Science **140**:997–998.
14. Gomatos, P. J., R. M. Krug, and I. Tamm. 1964. Enzymic synthesis of RNA with reovirus RNA as template. I. Characteristics of the reaction catalyzed by the RNA polymerase from *Escherichia coli*. J. Mol. Biol. **9**:193–207.
15. Gomatos, P. J., R. M. Krug, and I. Tamm. 1965. Reovirus RNA-directed synthesis of DNA. I. The reaction catalyzed by DNA polymerase from *Escherichia coli*. J. Mol. Biol. **13**:802–816.
16. Gomatos, P. J., I. Tamm, S. Dales, and R. M. Franklin. 1962. Reovirus type 3: physical characteristics and inter action with L cells. Virology **17**:441–454.
17. Koide, F., I. Suzuka, and K. Sekiguchi. 1968. Some properties of an adenine rich polynucleotide fragment from the avian reovirus. Biochem. Biophys. Res. Commun. **30**:95–99.
18. Krug, R. M., P. J. Gomatos, and I. Tamm. 1965. Enzymic synthesis of RNA with reovirus RNA as template. II. Nearest neighbor analysis of the products of the reaction catalyzed by the *Escherichia coli* RNA polymerase. J Mol. Biol. **12**:872–880.
19. Langridge, R., and P. J. Gomatos. 1963. The structure of RNA. Science **141**:694–698.
20. Loh, P. C., and A. J. Shatkin. 1968. Structural proteins of reoviruses. J. Virol. **2**:1353–1359.
21. Lowry, O.H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
22. Penman, S. 1966. RNA metabolism in the HeLa cell nucleus. J. Mol. Biol. **17**:117–130.
23. Shatkin, A. J. 1965. Inactivity of purified reovirus RNA as a template for *E. coli* polymerases *in vitro*. Proc. Nat. Acad. Sci. U.S.A. **54**:1721–1728.
24. Shatkin, A. J., and J. D. Sipe. 1968. Single-stranded, adenine-rich RNA from purified reoviruses. Proc. Nat. Acad. Sci. U.S.A. **59**:246–253.
25. Smith, J. D., and R. Markham. 1950. Chromatographic studies on nucleic acids. 2. The quantitative analysis of ribonucleic acids. Biochem. J. **46**:509–513.
26. Spendlove, R. S., and M. E. McClain. 1968. Enhancement and inactivation of reovirus infectivity by proteolytic enzymes, p. 127–130. *In* M. Sanders and E. H. Lennette (ed.), Medical and applied virology. Warren H. Green, Inc., St. Louis.