Synthesis of Reovirus Ribonucleic Acid in L Cells

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ABSTRACT

KUDO, HAJIME (The Wistar Institute of Anatomy and Biology, Philadelphia, Pa.), AND A. F. GRAHAM. Synthesis of reovirus ribonucleic acid in L cells. J. Bacteriol. 90:936–945. 1965.—There is no inhibition of protein or deoxyribonucleic acid (DNA) synthesis in L cells infected with reovirus until the time that new virus starts to form about 8 hr after infection. At this time, both protein synthesis and DNA synthesis commence to be inhibited. Neither the synthesis of ribosomal ribonucleic acid (RNA) nor that of the rapidly labeled RNA of the cell nucleus is inhibited before 10 hr after infection. Actinomycin at a concentration of 0.5 μg/ml does not inhibit the formation of reovirus, although higher concentrations of the antibiotic do so. Pulse-labeling experiments with uridine-C^{14} carried out in the presence of 0.5 μg/ml of actinomycin show that, at 6 to 8 hr after infection, two species of virus-specific RNA begin to form and increase in quantity as time goes on. One species is sensitive to ribonuclease action and the other is very resistant. The latter RNA is probably double-stranded viral progeny RNA, and it constitutes approximately 40% of the RNA formed up to 16 hr after infection. The function of the ribonuclease-sensitive RNA is not yet known. Synthesis of both species of RNA is inhibited by 5 μg/ml of actinomycin added at early times after infection. Added 6 to 8 hr after infection, when virus-specific RNA has already commenced to form, 5 μg/ml of actinomycin no longer inhibit the formation of either species of RNA.

Since the discovery by Montagnier and Sanders (1963) that replication of encephalomyocarditis (EMC) virus involves the synthesis of a double-stranded ribonucleic acid (RNA), similar double-stranded forms have been found during the multiplication of other single-stranded RNA viruses (Kelly and Sinsheimer, 1964; Weissmann et al., 1964; Erikson, Fenwick, and Franklin, 1964; Baltimore, Becker, and Darnell, 1964; Shipp and Haselkorn, 1964; Burdon et al., 1964). Considerable evidence is accumulating to suggest that these double-stranded, intermediate forms of RNA occupy a central role in the replication of single-stranded RNA viruses.

Such considerations raise a number of questions about the relationship between the replication of single-stranded RNA viruses and those that contain double-stranded RNA in the virion. Only one of the latter viruses is known which is infectious for mammalian cells, namely, reovirus, and its general properties have been extensively studied (Gomatos et al., 1962; Rhim, Jordan, and Mayor, 1962; Vasquez and Tournier, 1962; Jordan and Mayor, 1962; Gomatos and Tamm, 1963a, b, c). The virus is approximately 70 mμ in diameter and contains an amount of RNA equivalent to a molecular weight of 10^7. This RNA is extremely resistant to heat and to the action of ribonuclease and, in fact, has been shown by X-ray diffraction methods to have a double-helical structure very similar to that of the double-stranded intermediate form of MS2 phage (Langridge and Gomatos, 1963; Langridge et al., 1964).

Apart from the intrinsic interest in reovirus, therefore, a study of its mode of replication may help to cast some additional light on the function of the double-stranded intermediate form of the single-stranded RNA viruses. The present paper represents the beginning of a study of the multiplication of reovirus in L cells. In it are described some of the general features of RNA synthesis in the infected cells.

MATERIALS AND METHODS

Cells and medium. Strain L mouse fibroblasts were propagated in suspension (Homma and Graham, 1963) in a medium containing a twofold concentration of Eagle's (1959) basal mixture and Earle's salt solution modified to contain 10 times the standard concentration of phosphate and no calcium. Fetal calf serum was added to a concentration of 7%. For infection of L cells and growth of reovirus, the cells were centrifuged and suspended in a medium which contained four times the standard amount of vitamins and which was supplemented with 2% fetal calf serum.

Virus. Reovirus, type 3, Dearing strain, was obtained through the kindness of P. J. Gomatos and I. Tamm of The Rockefeller Institute. It had been cloned five times and then received one further passage in L cells (Gomatos and Tamm, 1963a). After receipt, the virus was passaged several times in suspension cultures of L cells. A culture was then infected at a multiplicity (*m*) of 20 plaque-forming units (PFU) per cell and incubated at 37 C. After 48 hr, the culture was frozen and thawed three times, and was centrifuged at 5,000 × *g* for 20 min. The supernatant solution contained 1.5×10^8 PFU and 1,000 hemagglutinating (HA) units per ml. It was kept at −70 C and was used as stock virus for the following experiments.

Plaque and hemagglutination assay. The plaque procedure of Gomatos et al. (1962) was slightly modified. Monolayers of L cells were cultivated in the standard growth medium, suspended with trypsin, and 10^6 cells were seeded into 50-mm plastic petri dishes. When the monolayers had been completely formed, 0.2 ml of diluted virus was added. After a 2-hr period for adsorption of the virus, each plate received 7 ml of an overlay consisting of equal volumes of 1.8% Noble agar (Difco) and twofold concentrated virus growth medium supplemented with 10% of fetal calf serum. The plates were incubated for 3 days at 37 C in an atmosphere of 5% CO_2-air, an additional 5 ml of the overlay were added, and they were incubated for an additional 3 days. A 5-ml amount of overlay medium containing 0.005% neutral red was then added, the plates were incubated for another 24 to 48 hr, and the plaques were counted.

Hemagglutination titrations of virus, with the use of ox erythrocytes, were carried out as described by Eggers, Gomatos, and Tamm (1962). The end point of the titration was taken to be the dilution of virus that showed a partial hemagglutination, and was expressed as the reciprocal of this dilution.

General procedure for infecting the cultures. L cells in the logarithmic phase of growth were centrifuged and suspended in the virus growth medium at a concentration of 5×10^5 cells per milliliter. These cultures were incubated in suspension at 37 C for 1 hr. In those experiments in which actinomycin D was used, an appropriate concentration of the drug was added, and the cultures were further incubated for 1 hr. Reovirus was then added at *m* = 8 to 10, and incubation of the culture at 37 C was continued.

Extraction of RNA from L cells. For extraction of RNA, a volume of culture (200 ml) containing 10^8 cells was centrifuged at 1,000 × *g* for 7 min, and the cells were washed once with cold 0.14 M NaCl. These cells were then stored at −70 C. RNA was extracted from the frozen cells either by use of a phenol-sodium dodecyl sulfate (SDS) procedure at 60 C, which extracted all the RNA of the cells (Scherrer and Darnell, 1962; Homma and Graham, 1963; Rake and Graham, 1964), or by use of a technique in which phenol-ethylene-

diaminetetraacetic acid (EDTA) at 4 C was used to extract the ribosomal and s-RNA of the cell, leaving behind the rapidly labeled nuclear RNA (Homma and Graham, 1963; Rake and Graham, 1964). In either case, the extracted RNA was precipitated with 6 volumes of ethyl alcohol, the precipitates were dissolved in 2 ml of buffer containing 0.14 M LiCl, 0.01 M tris(hydroxymethyl) aminomethane (Tris), *p*H 7.6, and 0.001 M $MgCl_2$ (LTM buffer), and were passed through a column of Sephadex G-25 gel (Homma and Graham, 1963). Those fractions which eluted in the outside volume of the column were combined, and the RNA was precipitated by the addition of ethyl alcohol.

For analysis by gradient centrifugation, the RNA was dissolved in LTM buffer, and 0.5 ml was layered over a 5 to 20% linear sucrose gradient. After centrifugation in the SW39 head of a Spinco model L centrifuge, three-drop samples were collected into 1 ml of LTM buffer. Optical densities (OD) at 260 m*μ* were taken, and each sample was subdivided into the required number of portions. As described later, ribonuclease (Worthington Biochemical Corp., Freehold, N.J.) was added to some of these portions at the desired concentration. Finally, one drop of 0.5% bovine albumin, and then an equal volume of cold 10% trichloroacetic acid, were added to all samples. The precipitates were collected on glass-fiber filters (Gelman Instrument Co., Ann Arbor, Mich.; type E), and their radioactivity was determined with a Tricarb scintillation spectrometer.

Purification of P^{32}-labeled reovirus. The purification procedure was somewhat modified from that described by Gomatos and Tamm (1963a, b). L cells were centrifuged and then suspended to a concentration of 10^6 per milliliter in 200 ml of virus growth medium from which phosphate had been omitted. After 1 hr at 37 C, the culture was infected at *m* = 20, and 2 hr later 2 mc of carrier-free P^{32} orthophosphate were added. Incubation was continued for 40 hr, after which the culture was frozen and thawed three times and centrifuged briefly at 5,000 × *g*. Ammonium sulfate was added to the supernatant solution to a concentration of 2 M. After several hours at 4 C, the resulting precipitate was centrifuged and dissolved in 15 ml of Tryptose Phosphate Broth (TPB) containing 30 *μ*g/ml of deoxyribonuclease and 60 *μ*g/ml of ribonuclease. The solution was kept at 37 C for 30 min, and 30 *μ*g/ml of crystalline trypsin were added. After a further short period of incubation, the preparation was dialyzed for 18 hr against 0.02 M phosphate buffer (*p*H 7.2). One-half volume of genetron-113 was added to the dialyzed solution, the mixture was shaken vigorously for 3 min, and the two phases were separated by centrifugation. The aqueous phase was again treated with genetron-113 and passed through a column of diethylaminoethyl (DEAE)-cellulose (10 by 1 cm in diameter). The virus was quantitatively adsorbed. A linear gradient of NaCl was applied to the column, and the virus was eluted at 0.3 M. HA

activity in the eluate coincided with a sharp, isolated peak of P^{32}. The several fractions containing the virus were combined, cesium chloride was added to an average density of 1.34, and the mixture was centrifuged at 36,000 rev/min in the SW39 head of a Spinco model L centrifuge for 24 hr. Three-drop fractions from the gradient were collected into TPB. A single peak of P^{32} coincided with the HA activity of the virus, as found by Gomatos and Tamm (1963a, b). Such purified preparations of virus contained approximately 2×10^{-4} counts per min per PFU, and had about 7×10^4 PFU per HA unit. No more than 3% of the P^{32} was acid-soluble. The overall recovery of purified virus was about 15% of the amount in the original lysate.

RESULTS

Overall synthesis of protein, RNA, and deoxyribonucleic acid (DNA) during infection. Using autoradiographic techniques, Gomatos and Tamm (1963c) showed that infection of L cells with reovirus does not inhibit the overall synthesis of RNA and protein up to the time of completion of virus formation, about 16 hr after infection. They reported that a marked inhibition of DNA synthesis occurs beginning at 8 to 9 hr. We have studied these synthetic processes by somewhat different means. Samples were withdrawn at intervals from two cultures of L cells, one of which had been infected at $m = 8$. Each sample was pulse-labeled for 30 min, with 0.05 μc of lysine-C^{14} and 0.5 μc of thymidine-H^3 added together per ml of culture. Another set of such samples was pulse-labeled for 2 hr with 0.05 μc of uridine-C^{14} per ml. After the period of labeling, measured volumes of each sample were mixed with an equal amount of cold 10% trichloroacetic acid, and the precipitates were filtered and assayed for radioactivity.

Measurements of HA activity in the infected culture of this experiment showed that virus began to form in the cells no later than 8 to 10 hr after infection, and continued until approximately 17 hr, in agreement with the results of Gomatos et al. (1962). The results with pulse-labeling (Fig. 1) indicate that there was little inhibition of protein synthesis or DNA synthesis up to the time virus commenced to form. After this time, there was some inhibition of protein synthesis and a marked inhibition of DNA synthesis. No inhibition of RNA synthesis was observed. However, as RNA synthesis was measured by uptake of uridine-C^{14}, there may have been incorporation of C^{14} into DNA, and some inhibition of RNA synthesis could have gone unobserved; this point will be considered in more detail later. In general, the results are similar to those of Gomatos and Tamm (1963c) except for
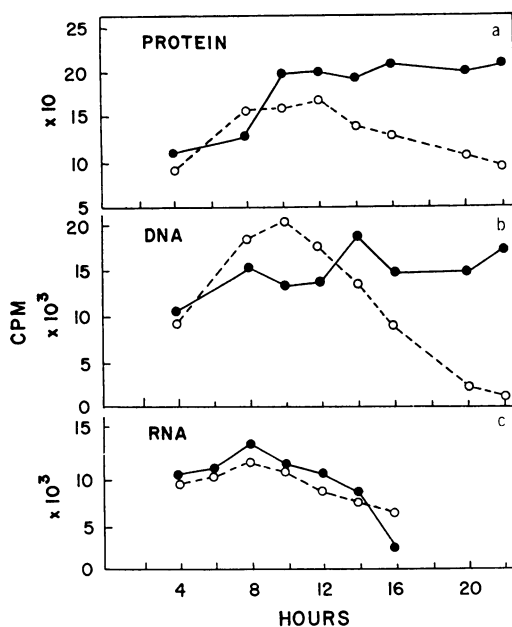

FIG. 1. *Synthesis of protein, DNA, and RNA after infection of L cells with reovirus. (a) Uptake of lysine-C^{14}; (b) uptake of thymidine-H^3; (c) uptake of uridine-C^{14}. Solid lines, uninfected control; broken lines, infected cells.*

our observation of a late inhibition of protein synthesis. In any event, during infection with reovirus there is no early inhibition of cellular RNA, DNA, and protein synthesis as there is with the smaller, virulent single-stranded RNA viruses (Franklin and Baltimore, 1962; Zimmerman, Heeter, and Darnell, 1963; Fenwick, 1963; Homma and Graham, 1963; Hausen and Verwoerd, 1963; Holland, 1964; Ellis and Paranchych, 1963).

Action of actinomycin on the formation of reovirus. To follow the formation of virus-specific RNA, it was clearly necessary to find a means of suppressing the synthesis of normal RNA in the infected cells. As is well known, synthesis of such cellular RNA can be inhibited with actinomycin in cells infected by many single-stranded RNA viruses without effect on virus multiplication (Franklin and Baltimore, 1962). In the case of reovirus, it has been shown by Gomatos et al. (1962) that viral growth is 95% inhibited by a concentration of 2 $\mu g/ml$ of actinomycin. This is a relatively high concentration of actinomycin, however, since it is known that 0.3 $\mu g/ml$ will completely block the synthesis of ribosomal RNA in L cells (Homma and Graham, 1962). Results of an experiment on the effect of different concentrations of actinomycin on virus production

TABLE 1. *Effect of actinomycin on multiplication of reovirus*

Concn actinomycin*	Virus yield at 48 hr after infection†	
	PFU/ml	HA/ml
μg/ml		
0	2.0×10^7	320
0.1	4.1×10^7	1,024
0.5	1.6×10^7	320
1.0	1.1×10^7	25

* Actinomycin was added 1 hr before infection at $m = 10$.

† Lysates were frozen and thawed three times before being assayed.

are shown in Table 1. Virus formation was not inhibited by 0.5 μg/ml of actinomycin. Multiplication of virus was inhibited by 50%, up to 80% in other experiments, by 1 μg/ml of actinomycin. (The low HA titer at 1 μg/ml in Table 1 is almost certainly the result of a technical error.) A slight increase in viral yield was regularly found at 0.1 μg/ml of antibiotic. These results are in agreement with those recently published by Shatkin (1965).

Effect of actinomycin on the latent period and the adsorption of reovirus to L cells. The results presented in Fig. 2 show that the presence of 0.5 μg/ml of actinomycin does not affect the length of the latent period. In this experiment, the cells were infected at $m = 10$. Extracellular virus and cell-associated virus (measured after freezing and thawing the infected cells three times) were determined both by plaque and HA assay. Virus commenced to form in the cells at approximately 8 hr, in agreement with the results of Gomatos et al. (1962) and of our own experiments carried out in the absence of actinomycin.

The adsorption rate of virus in this system, whether estimated by the adsorption of infectivity or of P^{32} from purified labeled virus, amounted to 50% in 1 hr. The general course of adsorption was similar to that described by Gomatos et al. (1962).

Uptake of uridine-C^{14} by infected cells in the presence of actinomycin. A suspension culture containing 5×10^5 cells per milliliter, to which 0.5 μg/ml of actinomycin had been added 1 hr before, was divided into two parts. Uridine-C^{14}, 0.05 μc per ml of culture, was added to both portions, and at the same time one part was infected by the addition of virus at $m = 10$. Samples were withdrawn from time to time, mixed with an equal volume of 10% trichloroacetic acid, and the resulting precipitates were filtered and assayed for C^{14}. The results (Fig. 3) indicate that the in-

corporation of C^{14} into both infected and uninfected cultures proceeded in parallel for some 4 to 5 hr. Further incorporation into the uninfected culture then stopped, while the infected culture continued to take up C^{14}. The early incorporation of C^{14} by both cultures was into s-RNA as will be seen in a later section. After 5 hr, however, the uptake into infected cells represented the formation of a new RNA, as Shatkin (1965) has also found. Thus, an RNA formed specifically by virus-infected cells could be detected some 2 hr before viral maturation since, in this experiment, new virus was first found about 8 hr after infection.

Sucrose gradient analysis of RNA extracted from infected cells. In view of the results presented above, the following experiment was done to analyze the distribution of C^{14} in RNA formed at different times after infection. A large culture was infected at $m = 10$ in the presence of 0.5 μg/ ml of actinomycin. At 0, 2, 4, 6, 8, and 10 hr, 200-ml samples were withdrawn and incubated for an additional 2 hr with 5 μc of uridine-C^{14}. After this 2-hr pulse-labeling period, the RNA was extracted by the phenol-SDS procedure, and was centrifuged for 2 hr at 36,000 rev/min through a sucrose gradient. Each fraction from the gradient was assayed for OD at 260 mμ and for trichloroacetic acid-precipitable C^{14} before and after the
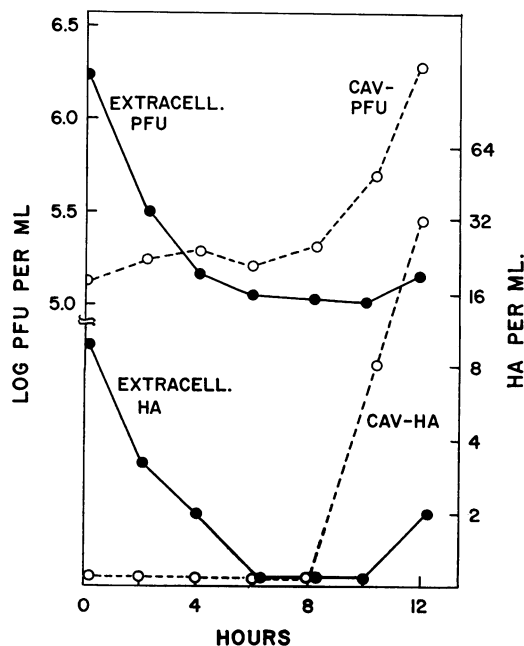

FIG. 2. *Latent period and adsorption of reovirus to L cells in the presence of 0.5 μg/ml of actinomycin. CAV = cell-associated virus.*

action of 2 μg/ml of ribonuclease for 30 min at 37 C. The essential results are shown in Fig. 4. Up to 6 hr after infection, the only C^{14} taken up by RNA appeared in the 4S (s-RNA) fraction. C^{14} then began to be incorporated into a new and
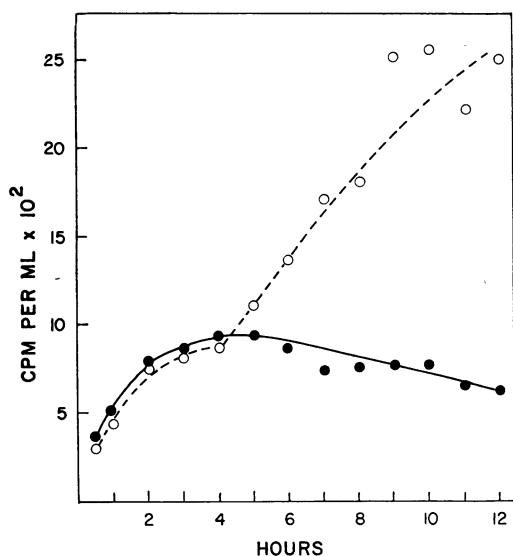

FIG. 3. *Uptake of uridine-C^{14} in the presence of 0.5 μg/ml of actinomycin. Broken line, infected cells; solid line, uninfected cells. The uridine was added at time-zero.*

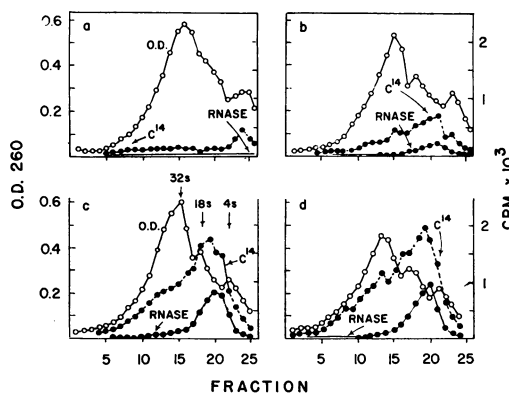FIG. 4. *Uptake of uridine-C^{14} into ribonuclease-sensitive and ribonuclease-resistant RNA in infected cells. Actinomycin (0.5 μg/ml) was added to the cultures at minus 1 hr, and reovirus at time-zero. The cultures were labeled at (a) 4 to 6 hr, (b) 6 to 8 hr, (c) 8 to 10 hr, and (d) 10 to 12 hr. Analyses were by sucrose gradient sedimentation at 36,000 rev/min for 2 hr. C^{14} represents the total amount of C^{14} precipitated by trichloroacetic acid and RNASE represents the C^{14} precipitated trichloroacetic acid after the action of 2 μg/ml of ribonuclease.*

TABLE 2. *Incorporation of uridine-C^{14} into the RNA fractions of infected cells*[*]

Labeling period (hr after infection)	Total RNA-C^{14}/OD unit	Ribonuclease-resistant RNA-C^{14}/OD unit	Ratio of ribonuclease-resistant RNA-C^{14} to total RNA-C^{14} × 100
	count/min	count/min	
0–2	1,100	0	0
2–4	800	0	0
4–6	500	0	0
6–8	1,400	200	14
8–10	2,900	700	24
10–12	3,400	1,000	30

[*] Figures were calculated from the experimental data in Fig. 4.

heterogeneous fraction of RNA that sedimented between 3S and 35S approximately. A small part of this new RNA, which sedimented between 4S and 20S, was ribonuclease-resistant. As time went on, an increasing amount of label was taken up both by the ribonuclease-sensitive and ribonuclease-resistant fractions. The incorporated label was estimated by summing the amounts of C^{14} appearing in the various gradients and dividing these figures by the total OD found in the respective gradients. These results are shown in Table 2 for the four gradients of Fig. 5 and for two other analyses carried out in the same experiment but not shown in Fig. 5. In agreement with the data of Fig. 3, incorporation of C^{14} into new RNA started between 6 and 8 hr. At approximately the same time, the ribonuclease-resistant RNA began to form, and this fraction amounted to 30% of the total amount of RNA that became labeled between 10 and 12 hr. To ensure that virus was being formed during this experiment, assays of PFU and HA were carried out from time to time (Fig. 2).

Separation of ribonuclease-resistant RNA of infected cells from the ribonuclease-sensitive RNA. For the purpose of further investigating the ribonuclease-resistant and ribonuclease-sensitive RNA formed in infected cells, it was necessary to have methods for separating the two fractions. Three such methods are described in the following paragraphs.

Use of different phenol extraction techniques. Whereas the phenol-SDS procedure completely extracts the RNA from L cells, extraction with phenol in the cold, against an aqueous phase containing EDTA and no SDS, does not remove the rapidly labeled RNA of the cell nucleus (Rake and Graham, 1964). The same two phenol methods can be utilized to separate the ribonuclease-sensitive and -insensitive RNA formed during

reovirus infection, as the following experiment shows.

A large culture of L cells, to which actinomycin had been added to 0.5 μg/ml, was divided, and one part was infected with reovirus at $m = 10$. After 2 hr, 5 μc of uridine-C^{14} per 200 ml of culture were added. At 13 hr after infection, several 200-ml samples were taken for extraction of RNA. Figure 5a shows a sucrose gradient analysis of RNA extracted from the uninfected culture by phenol-SDS; most of the incorporated C^{14} appeared in the 4S fraction, as suggested in an earlier section, and it was all sensitive to 2 μg of ribonuclease per ml at 37 C for 30 min. An analysis of RNA extracted from the infected culture by the same technique is shown in Fig. 5b. One peak of C^{14} is centered at 32S, another and much larger peak appears at approximately 15S, and a third peak at 4S is the s-RNA. After the action of 2 μg/ml of ribonuclease, for 30 min at 37 C, a single large peak at 15S is seen. It should be emphasized that addition of deoxyribonuclease (100 μg/ml at 37 C for 30 min) to the various fractions did not change the C^{14} profiles.

Another portion of the infected culture was extracted by the phenol-EDTA method; the sedimentation analysis of this RNA is shown in Fig. 5c. The material remaining at the interface between the phenol and aqueous phases was then extracted again with phenol-SDS; the analysis of this extract is shown Fig. 5d. Fig. 5c shows clearly that most of the labeled RNA extracted by phenol-EDTA was the ribonuclease-resistant 15S material of Fig. 5b. Of the C^{14} contained in the 15S peak (Fig. 5c), 78, 61, and 8% remained acid-insoluble after the action of 2, 20, and 200 μg, respectively, of ribonuclease per ml for 30 min at 37 C; this RNA is extraordinarily resistant to the action of ribonuclease (Gomatos, Krug, and Tamm, 1964). As shown in Fig. 5d, the second extraction with phenol-SDS then removed the ribonuclease-sensitive material, although not quantitatively. Further work is needed to improve the efficiency of this second extraction, but it is clear that a fairly clean separation of the two classes of RNA can be made with the two phenol procedures.

Effect of magnesium ion concentration. Mg ion concentration affects the sedimentation rate of the single-stranded RNA of poliovirus (Zimmerman et al., 1963), but has little influence on sedimentation of the double-stranded intermediate form of EMC virus (Montagnier, *personal communication*). To determine the effect of Mg ion on the sedimentation rate of RNA from reovirus-infected cells, one of the portions of labeled cells described in the preceding section was extracted with phenol-SDS. After passage through a
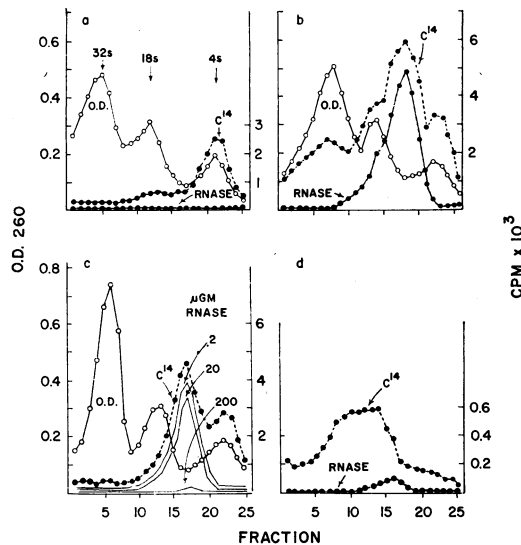

FIG. 5. *Separation of ribonuclease-sensitive RNA of infected cells from ribonuclease-resistant RNA by two phenol extraction procedures. Sucrose gradient sedimentation analyses were of (a) RNA extracted from uninfected cells with phenol-SDS; (b) RNA extracted from infected cells by phenol-SDS; (c) RNA extracted from infected cells by phenol-EDTA, and showing the action of different concentrations of ribonuclease; and (d) RNA extracted by phenol-SDS from the interfacial material left after the phenol-EDTA extraction. All cultures contained 0.5 μg/ml of actinomycin and were labeled with uridine-C^{14} between 2 and 13 hr after infection. Sedimentation was at 36,000 rev/min for 4 hr. C^{14} represents the total amount of C^{14} in each fraction, and RNASE represents the C^{14} precipitated by trichloroacetic acid after the action of ribonuclease. Deoxyribonuclease (100 μg/ml) did not change C^{14} profiles in b.*

Sephadex column and precipitation with ethyl alcohol, samples of the RNA were dissolved in LTM buffer containing different concentrations of Mg ion and then analyzed by sedimentation of sucrose gradients containing the respective concentrations of Mg. In the absence of Mg ion, ribosomal RNA (and the ribonuclease-sensitive, C^{14}-labeled RNA) trailed behind ribonuclease-resistant RNA. At a concentration of 10^{-1} M Mg ion, the sedimentation rate of ribonuclease-resistant RNA was essentially unchanged, but the remaining RNA, with the exception of the 4S fraction, went to the bottom of the tube.

Chromatography on a column of methylated albumin. A further portion of the infected, labeled cells (experiment of Fig. 5) was extracted by the phenol-SDS procedure, and the RNA was dissolved in 0.1 M NaCl-0.05 M phosphate buffer

(pH 7.6) and applied to a column of methylated bovine albumin (Sueoka and Cheng, 1962; Homma and Graham, 1963). The column was developed with a linear gradient of NaCl, with the mixing chamber containing 100 ml of 0.1 M NaCl and the reservoir chamber 2 M NaCl (Fig. 6). Ribonuclease-resistant RNA was eluted at approximately 0.78 to 0.82 M NaCl, just before ribosomal RNA, and in almost the same position found for the double-stranded form of poliovirus (Pons, 1964). Ribonuclease-sensitive RNA was eluted much later than the resistant form and, clearly, was not removed completely from the column.

Identification of the ribonuclease-resistant RNA in infected cells as viral RNA. Intensive work on reovirus RNA has shown that, among other properties, it is ribonuclease-resistant and double-stranded (Gomatos and Tamm, 1963a, b; Langridge and Gomatos, 1963). From the experiments described above, it seemed almost certain that the ribonuclease-resistant RNA formed in the infected cells was the viral progeny RNA. The following experiments support this supposition. Infected, labeled cells (experiment of Fig. 5) were extracted by the phenol-EDTA method, and the RNA was analyzed on a sucrose gradient. Material from the C^{14}-labeled peak (see Fig. 5c) was mixed with the RNA extracted by phenol-SDS from cells that had adsorbed purified, P^{32}-labeled virus for 1 hr at $m = 2$. This mixture was analyzed by gradient sedimentation with the results shown in Fig. 7. It is clear that the peaks of P^{32}-labeled RNA and C^{14}-labeled RNA coincided and that they were equally resistant to ribonuclease.

A similar result was obtained when RNA was extracted from P^{32}-labeled purified virus before adsorption to the host cells. Thus, the ribonu-
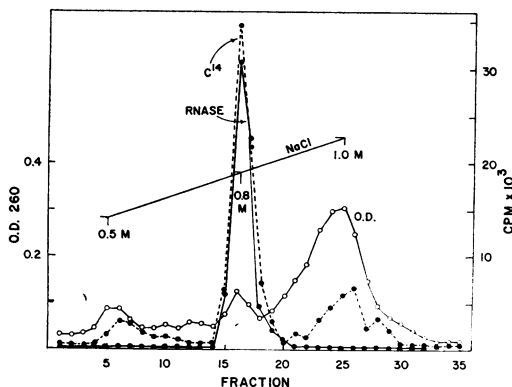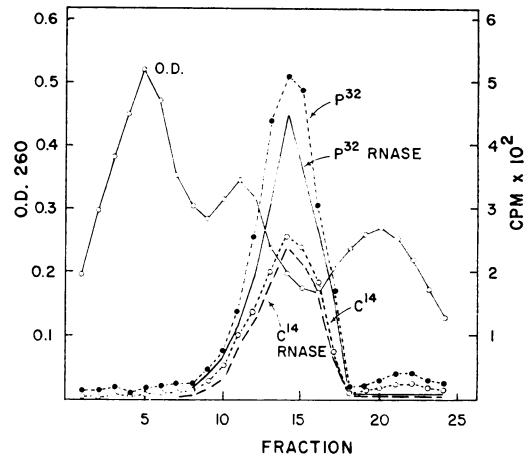

FIG. 7. *Sedimentation analysis of RNA extracted from cells that had adsorbed P^{32}-labeled, purified virus in admixture with the ribonuclease-resistant, C^{14}-labeled RNA from infected cells. Sedimentation was at 36,000 rev/min for 4 hr. C^{14} and P^{32} represent the amount of labeled material in each fraction and C^{14} RNASE and P^{32} RNASE represent the amount of labeled material precipitated by trichloroacetic acid after the action of 2 μg/ml of ribonuclease.*

clease-resistant RNA formed in infected cells would seem to be largely viral progeny RNA. Furthermore, the results suggest that the ribonuclease-sensitive RNA found in the earlier experiments was not an artifact produced by the breakdown of viral RNA during extraction.

Effect of actinomycin on the synthesis of virus-specific RNA. When L cells were treated with 1 to 2 μg/ml of actinomycin before infection, or at the time of infection, the yield of virus was much reduced, as shown by Gomatos et al. (1962) and Shatkin (1965), and by the results in Table 1 of the present paper. Gomatos et al. (1964) also found that the same concentrations of actinomycin inhibit the in vitro synthesis of RNA primed by reovirus RNA and catalyzed by an RNA polymerase from *Escherichia coli*. These results suggested an experiment to determine whether high concentrations of actinomycin, added some time after infection, would inhibit the further formation of virus-specific RNA.

Five 200-ml suspension cultures were kept for 1 hr with a concentration of 0.5 μg/ml of actinomycin. Each culture was then infected at $m = 10$. At 2, 4, 6, and 8 hr after infection, additional actinomycin was added to one culture to give a final concentration of 5 μg/ml. One of the five cultures was maintained with the original concentration of actinomycin. A sixth culture received 5 μg/ml of actinomycin 1 hr before infection, and the same concentration was kept

FIG. 6. *Elution of ribonuclease-resistant and -sensitive RNA from a column of methylated bovine albumin by a linear NaCl gradient.*

throughout the experiment. At 9 hr after infection, 5 μc of uridine-C14 were added to each of the six cultures. After an additional 7 hr, the RNA was extracted from each culture by the phenol-SDS method and analyzed by gradient sedimentation. There was virtually no label in the s-RNA in any of the gradients, and the C14 profiles could then be considered to be due entirely to virus-specific RNA. The total C14 and the total ribonuclease-resistant C14 (2 μg/ml of ribonuclease for 30 min at 37 C) were computed for each analysis, and were divided by the total number of OD units in the respective gradients (Fig. 8).

When added 1 hr before infection, 5 μg/ml of actinomycin reduced the amount of RNA labeled to about 30% of that in the control culture containing 0.5 μg/ml. Mature virus formation was inhibited to about the same extent under the same conditions, as Gomatos et al. (1962) have found. Incorporation of C14 was not inhibited when the actinomycin was added at 8 hr, that is, at about the time that virus-specific RNA started to form in the cells. Apparently an early stage in the replication of viral RNA is sensitive to the inhibitory action of the antibiotic. Some 35 to 40% of the RNA-C14 in each culture was ribonuclease-resistant. Thus, incorporation of C14 into the total virus-specific RNA and incorporation into the ribonuclease-resistant fraction were equally sensitive to actinomycin inhibition, suggesting some close link between the synthesis of the two classes of RNA.

Synthesis of cellular RNA during infection. The experiment of Fig. 1 had suggested that infection with reovirus exerted little effect on the synthesis of cellular RNA. This question was investigated further in the following experiment. Three cultures were infected at m = 10, and 2 hr later uridine-C14 was added to each to a concentration of 0.025 μc/ml. By use of the phenol-EDTA method, RNA was extracted from one culture at each of 4, 9, and 12 hr after infection. Three uninfected cultures were labeled for similar intervals and extracted in the same way. Each specimen of RNA was analyzed by gradient sedimentation. It was difficult to make a precise estimate from the sedimentation patterns at 12 hr, since viral-specific RNA was present, but it was clear that there was practically no inhibition of ribosomal RNA synthesis before 9 hr and very little at 12 hr. Further experiments were done to determine the effect of virus infection on the formation of the rapidly labeled nuclear RNA (Rake and Graham, 1964). Uninfected and infected cells were pulse-labeled for 30 min with uridine-C14 at various times, and the RNA was extracted by the phenol-SDS method and analyzed by gradient
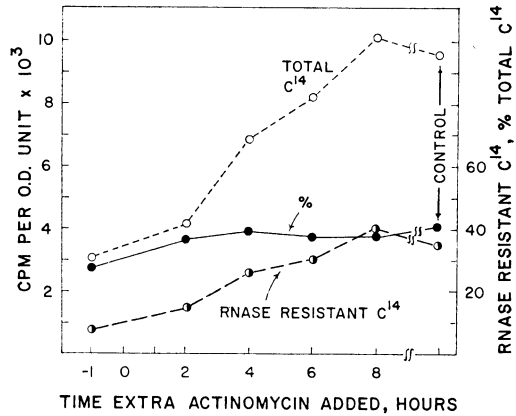

FIG. 8. *Effect of high concentrations of actinomycin, added at different times, on the uptake of uridine-C14 into virus-specific RNA. All cultures contained 0.5 μg/ml of actinomycin from 1 hr before infection; its concentration was increased to 5 μg/ml at the times shown by the points. The control culture contained 0.5 μg/ml of actinomycin throughout the experiment. Percentage represents the proportion of ribonuclease-resistant RNA-C14 to total RNA-C14. Uridine-C14 was present in each culture between 9 and 16 hr after infection.*

sedimentation. There was little or no inhibition of the synthesis of the nuclear RNA up to 12 hr after infection.

DISCUSSION

Some 6 hr after infection with reovirus, new RNA commenced to form in the cells. This RNA could be easily distinguished, since the synthesis of cellular RNA was inhibited with actinomycin. Two species of new RNA were formed. The first, because of its resistance to ribonuclease, its sedimentation rate compared with that of RNA extracted from reovirus, its solubility in 0.1 M Mg++, and its elution pattern from a column of methylated bovine albumin, was almost certainly viral progeny RNA. The second species comprised up to 60% of the total RNA synthesized and, being sensitive to ribonuclease, was probably single-stranded.

The sedimentation rate of the viral RNA, which averaged about 15S, was much lower than would be expected for a double-stranded molecule with a weight of 10^7 (Gomatos and Tamm, 1963a, b). It has been shown, however, by Gomatos and Stoeckenius (1964) and by Kleinschmidt et al. (1964) that, on extraction of RNA from reovirus, the molecule is broken into short lengths of which the longest represents a molecular weight of 3 × 10^6 to 4 × 10^6. The slow sedimentation rate observed here for the reovirus RNA is consistent

with that found by Gomatos and Stoekenius (1964), and is explained by breakdown of RNA into smaller fragments.

The single-stranded RNA formed after infection was heterogeneous and contained material sedimenting between 4S and 35S. The sedimentation patterns suggested that there may be at least two fairly distinct fractions of this RNA, but this point bears further investigation. As yet we have no information about the function of the single-stranded RNA, but there is a variety of obvious possibilities. It could be, (i) a precursor of viral RNA, (ii) a messenger RNA formed in direct response to the information supplied by the parental double strand of RNA to provide for the synthesis of new enzymes, or (iii) a messenger RNA to be utilized in the synthesis of viral coat protein. At least, it would appear not to be formed by the breakdown of double-stranded viral RNA during the phenol extraction. Probably the single-stranded RNA has several functions, but the suggested possibilities are open to immediate test.

Both the work of Gomatos et al. (1962) and the present work have shown that early addition of high concentrations of actinomycin (2 μg/ml) suppresses the formation of reovirus, although this inhibition is far from complete. Our results suggest that the block is in the synthesis of RNA. However, we find that, once virus-specific RNA has commenced to form in the cells, it is no longer inhibited even by 5 μg/ml of actinomycin. The action of the antibiotic is much earlier than on the major synthesis of virus-specific RNA. It has not yet been precluded, although it seems unlikely, that DNA participates early in the process of infection. Possibly, also, by the time virus-specific RNA can first be detected in the cells, it is enclosed in cytoplasmic structures (Rhim et al., 1962) that are impermeable to actinomycin. Most probably, as suggested by the results of Gomatos et al. (1964), actinomycin inhibits the formation of an early RNA produced by a pre-existing cellular polymerase which is primed by the parental RNA.

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