RNA POLYMERASE ACTIVITY IN PURIFIED REOVIRUSES

By A. J. Shatkin* and J. D. Sipe

LABORATORY OF BIOLOGY OF VIRUSES, NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

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The genome of reoviruses is double-stranded RNA.[1] Since helical RNA's,[2, 3] including reovirus RNA,[4, 5] have little or no messenger activity, it was of interest to examine how reoviruses initiate replication. In infected cells the first reovirus-specific, single-stranded messenger could result by transcription of the parental genome or by separation of its two strands. Attempts to demonstrate intracellular strand separation have been unsuccessful,[6, 7] and cellular polymerases which can utilize double-stranded reovirus RNA as template have not been detected.[8] However, an enzyme capable of catalyzing the synthesis of virus-specific, single-stranded RNA *in vitro* has been found associated with purified reoviruses. The characteristics of this RNA polymerase activity are described in this article.

Materials and Methods.—Crystalline α-chymotrypsin was purchased from C. F. Boehringer; twice-crystallized trypsin, papain, and lysozyme and electrophoretically purified DNase I and pancreatic RNase were obtained from the Worthington Biochemical Corporation. Pyruvate kinase and nucleoside triphosphates were products of Calbiochem. Radioactive nucleoside triphosphates and P^{32}-orthophosphate were from Schwarz BioResearch, Inc., and Tracerlab, respectively. Reovirus types 1 (Lang), 2 (D-5 Jones), and 3 (Abney) were obtained from the American Type Culture Collection.

The procedures for the growth of mouse L-929 cells, multiplication and purification of reoviruses, phenol extraction of viral RNA, annealing of single- and double-stranded RNA, and analysis of RNA by polyacrylamide gel electrophoresis have been described previously.[8-10] L-cell DNA was isolated by the Marmur method.[11] Protein was measured by the Lowry reaction.[12] Actinomycin D was kindly provided by Merck and Company.

For assay of RNA synthesis, the standard incubation mixture of 0.25 ml contained 0.5 μmole each of adenosine 5'-triphosphate (ATP), cytidine 5'-triphosphate (CTP), guanosine 5'-triphosphate (GTP), and uridine 5'-triphosphate (UTP), one of which was H^3-labeled at a final specific activity of 4–11 μc/μmole; 16 μmoles tris-HCl, pH 8.0; 3.2 μmoles magnesium acetate; 3.2 μmoles 2-mercaptoethanol; 0.7 μmole phosphoenolpyruvate (PEP); 25 μg (6 units) pyruvate kinase; and purified reovirus equivalent to 0.2–0.3 mg of protein (except where noted). After incubation for 40 min at 35°C, the reaction was stopped by the addition of 1 ml of 5% trichloroacetic acid (TCA) at 4°C. The precipitates were collected on nitrocellulose filters, washed with 5% TCA, dried, and counted in liquifluor–toluene at an efficiency of 25%.

Results.—Purified reovirus type 3 incubated under standard conditions at pH 7.5–9.0 did not incorporate H^3-cytidine 5'-phosphate (CMP) into an acid-insoluble product (Fig. 1). Since poxviruses contain an RNA polymerase which is demonstrable only after the particles are structurally altered,[13, 14] the effect of various treatments on the polymerase activity of reovirus was tested. Virus which had been exposed to ultrasound before incubation remained inactive. However, virus incubated together with chymotrypsin, an enzyme previously shown to increase the infectivity of reoviruses severalfold,[17] was highly active (Fig. 1). The optimal pH range for incorporation of radioactivity was 8.0–9.0, and no RNA synthesis occurred at pH 7.0. At pH 8.0, under standard condi-

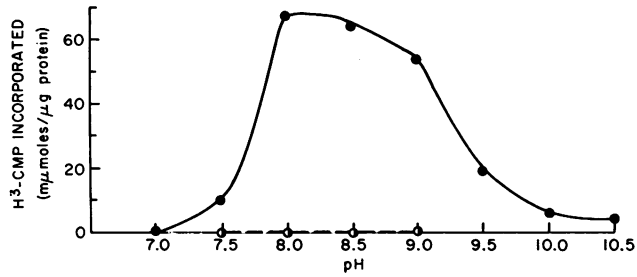


FIG. 1.—Dependence of polymerase activity on pH. Virus was incubated in the standard assay in the presence of 50 μg/ml of chymotrypsin (●) or in the absence of proteolytic enzyme before and after sonication (◑) at 10 kc for 2 min at 4°C in 0.01 *M* tris(hydroxymethylaminomethane (tris) buffer, pH 7.5.

tions, the presence of 50 μg/ml of chymotrypsin was required for maximum activity (Fig. 2). Increasing the concentration to 250 μg/ml decreased the incorporation by only 12 per cent, an indication that the activity is resistant to chymotryptic digestion.

As shown in Table 1, the reaction depends completely upon the presence of all four ribonucleoside triphosphates. Deletion from the incubation mixture of the ATP-generating system reduced the extent of synthesis, but 2-mercaptoethanol was not essential. Mg^{++} was required and not replaced by the same concentration of Mn^{++}. As in the case of the poxvirus-associated RNA polymerase,[13] 0.01 *M* phosphate inhibited the reaction completely. However, unlike the DNA-mediated reaction of poxviruses, actinomycin had no effect on the reovirus-associated activity, nor did DNase, L-cell DNA, or type 3 reovirus double-stranded RNA. When the deoxyribonucleoside triphosphates, including H^3-thymidine 5′-triphosphate (TTP) at a specific activity of 4 μc/μmole, were substituted for the ribose derivatives, no incorporation occurred.

Polymerase activity was not limited to chymotrypsin-treated type 3 virus. Incubation of 0.05–0.1 mg of types 1 and 3 virus for 120 minutes in the standard assay in the presence of 40 μg/ml of trypsin resulted in the incorporation of 75 and 206 mμmoles H^3-GMP/mg protein, respectively. Type 1 virus similarly

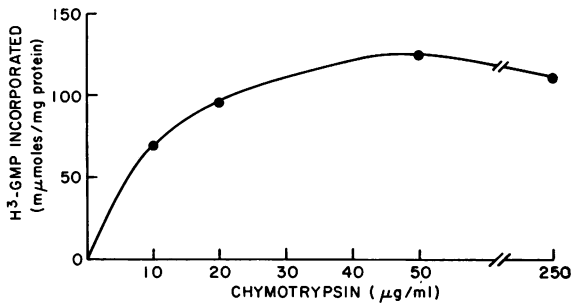


FIG. 2.—Effect of chymotrypsin concentration on RNA synthesis by type 3 virus incubated under standard assay conditions.

TABLE 1. *Characteristics of reovirus-associated RNA polymerase activity.*

Reaction mixture*	H^3-CMP incorporated (mμmoles/mg protein)	H^3-GMP incorporated (mμmoles/mg protein)
Complete	70.5	77.8
−Virus	0.6	0.2
−Chymotrypsin	0.6	0.8
−UTP	0.5	—
−GTP	0.7	—
−ATP	0.5	—
−CTP	—	0.3
−PEP kinase	38.8	60.7
−Mg	—	0.3
−Mg + Mn	—	0.8
−2-mercaptoethanol	66.5	—
+0.01 *M* PO_4 buffer, pH 8	0.3	—
+Actinomycin 5 μg/ml	66.5	—
" 10 μg/ml	88.4	—
+DNase 25 μg/ml	65.5	72.0
+L-cell DNA 7 μg	63.7	—
+Reovirus RNA 8 μg	67.5	—

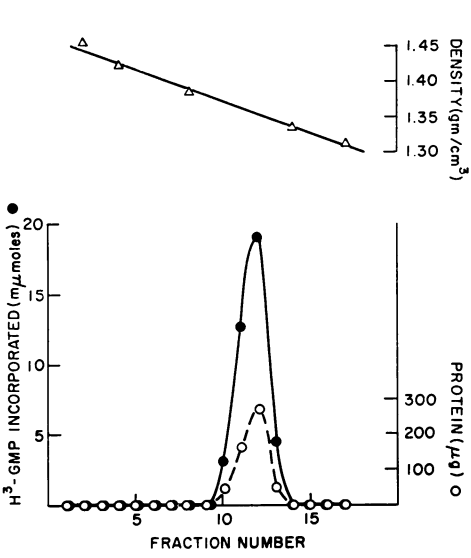
* Standard assay conditions with type 3 virus and 50 μg/ml of chymotrypsin present except where noted.

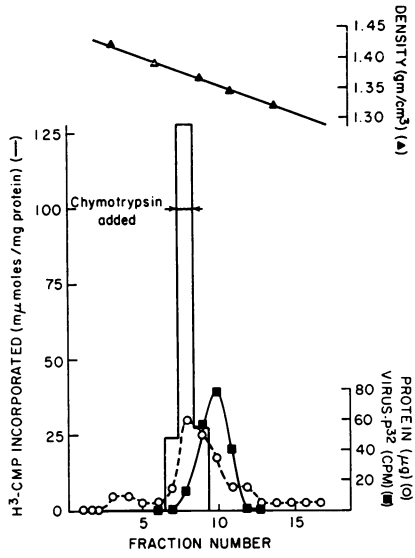
incubated with 4 μg/ml of chymotrypsin incorporated 28 mμmoles/mg. Exposure of type 1 to 4 μg/ml of papain or lysozyme resulted in small but significant amounts of synthesis, i.e., from two to three times the levels obtained without proteolytic enzymes. However, no activity was demonstrable under these various conditions with type 2 virus.

The RNA-synthesizing enzyme(s) apparently is an integral part of the virion since it was not separated from the particles by isopycnic sedimentation in CsCl (Fig. 3). Both the maximum specific activity of enzyme and the largest quantity of virions as measured by virus protein content had a buoyant density of 1.36 gm/cm.[3] Particles which are treated with chymotrypsin before centrifugation also sedimented with the polymerase activity but at a buoyant density of 1.38 gm/cm[3] as compared to 1.36 gm/cm[3] for the P^{32}-labeled virus which was added as a marker (Fig. 4). Incubation of the purified activated particles in the standard assay resulted in RNA synthesis without addition of chymotrypsin; in its presence 80 per cent of the maximum incorporation was observed, again indicating that the polymerase is resistant to inactivation by proteolytic enzymes.

The reovirus particle consists of two structural protein layers which enclose a core of RNA.[15, 16] It has been shown previously that the outer layer can be removed by treatment with chymotrypsin, a process accompanied by a marked rise in infectivity.[17] The increased buoyant density of the activated particles in Figure 4 is consistent with a similar structural alteration of the whole virions. Electron microscopic examination confirmed that the whole virions have a diameter of 65 mμ and consist of two protein coats. However, the denser, activated particles were only 45 mμ in diameter and lacked the outer layer of capsomeres (Fig. 5).

The kinetics of *in vitro* RNA synthesis were examined with chymotrypsin-activated, purified type 3 particles. The incorporation of H^3-GMP into acid-





(*Left*) FIG. 3.—Isopycnic sedimentation of type 3 virus in CsCl. After centrifugation to equilibrium, fractions were collected and dialyzed against 0.01 *M* tris buffer, pH 7.5. Aliquots were then assayed for protein content[12] and RNA polymerase activity in the presence of 50 μg/ml of chymotrypsin.

(*Right*) FIG. 4.—Sedimentation in CsCl of chymotrypsin-treated type 3 virus. After incubation of 1.2 mg type 3 virus for 40 min at 35°C with 50 μg/ml of chymotrypsin in 0.2 ml of 0.01 *M* tris buffer, pH 7.5, CsCl solution was added to a final volume of 4 ml and a density of 1.37 gm/cm³. Type 3 virus labeled with P^{32}-orthophosphate was then added (9000 cpm in 0.05 ml). After centrifugation for 24 hr (Spinco SW-39 rotor; 33,000 rpm), fractions were collected and dialyzed. Aliquots were assayed for radioactivity, protein content, and polymerase activity without chymotrypsin. Peak fraction number 8 was also assayed in the presence of 50 μg/ml of chymotrypsin.

insoluble product was directly proportional to the quantity of particles present in the incubation mixture (Fig. 6). The time course of the reaction is shown in Figure 7. Incorporation began with no detectable lag and continued for at least three hours. The rate of synthesis decreased by about 30 per cent during the last 90 minutes of incubation. After three hours' incubation, 8.2 mμmoles of H^3-GMP were incorporated into acid-insoluble material. Assuming that the product, like double-stranded viral RNA, contained 24 per cent GMP,[9, 18] it corresponded to 32.8 mμmoles of RNA nucleotides synthesized by an amount of particles containing 9 μg protein and 0.7 μg RNA, or 0.5 mμmole of RNA nucleotides. Thus the product represented a 60-fold increase over the template RNA and was probably released from the particles.

The size of the *in vitro* RNA product was compared to that of RNA extracted from purified type 3 virus. Heat-denatured, P^{32}-labeled type 3 virus RNA was mixed with H^3-labeled reaction product, and the mixture was analyzed by polyacrylamide gel electrophoresis (Fig. 8). As described previously,[10, 18, 19] the virus RNA-P^{32} separated into large (*L*), medium (*M*), and small (*S*) segments and an adenine-rich single-stranded ribopolymer (*A*) whose molecular weight, estimated from sedimentation measurements and gel analysis, is 4500–5300.[18]

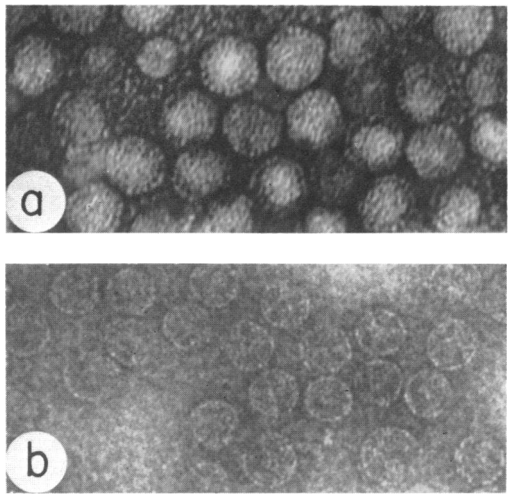


FIG. 5.—Electron micrographs of purified type 3 reovirus before (*a*) and after (*b*) treatment with chymotrypsin. Phosphotungstate-stained. ×133,000.

Most of the *in vitro* product migrated with *A;* but about 5 per cent of the total moved more slowly, forming a broad peak ahead of the *S* fragments. The molecular weight of RNA is directly proportional to mobility in polyacrylamide gels,[10, 20] and on this basis molecular weights of about 5,000 and 100,000 were estimated for the major and minor components of the reaction product, respectively.

Although the *in vitro* product was small relative to the reovirus genome segments, experiments with a highly specific annealing technique[9] indicate that it was copied from the double-stranded RNA. H^3-labeled RNA was synthesized with chymotrypsin-treated purified type 3 particles. Reovirus-specific, single-

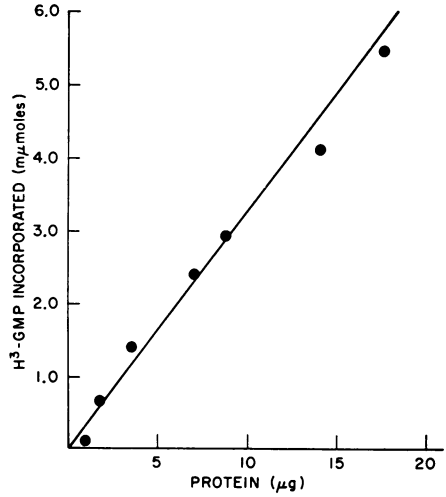


FIG. 6.—Dependence of RNA synthesis on particle concentration. Virus was treated with chymotrypsin; activated particles were then purified as in Fig. 4 and assayed in the standard reaction without chymotrypsin.

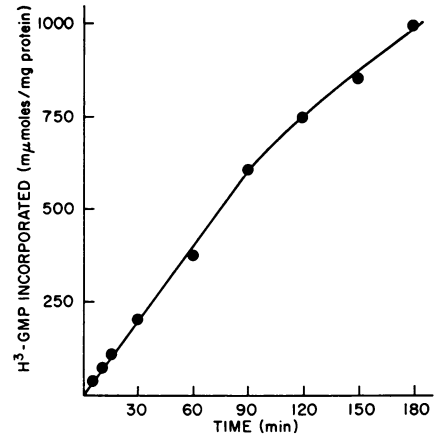


FIG. 7.—Time course of synthetic reaction. Chymotrypsin-treated particles were purified by centrifugation in CsCl. Aliquots were incubated for the indicated intervals in the standard assay without addition of chymotrypsin.

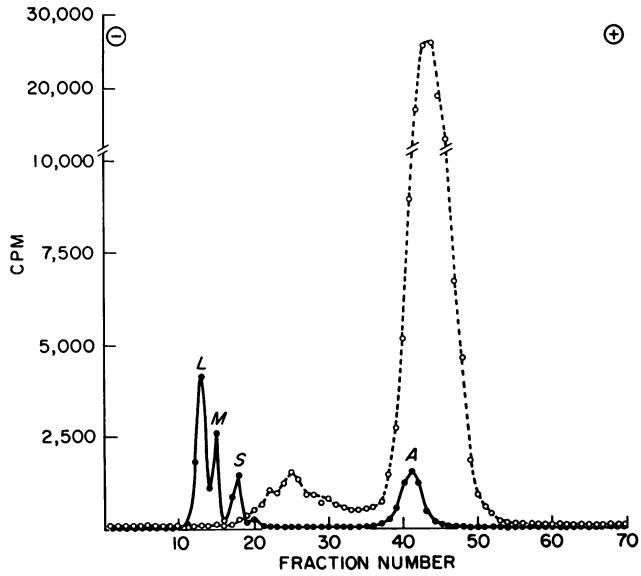


FIG. 8.—Comparison of the sizes of *in vitro* reaction product and virus RNA by polyacrylamide gel electrophoresis. After 30 min incubation of 0.48 mg purified type 3 virus in 0.5 ml of standard assay mixture containing H^3-GTP and 20 μg chymotrypsin, the reaction was stopped by chilling and the addition of an equal volume of 0.01 *M* tris buffer, pH 7.4, containing 0.3 *M* NaCl and 0.001 *M* ethylenediaminetetraacetate (EDTA). This was followed by 1 ml H_2O-saturated phenol and sodium dodecylsulfate to a final concentration of 0.5%. The mixture was gently shaken at room temperature for 5 min and centrifuged briefly to separate the phases. RNA was precipitated from the aqueous phase by adding 2 vol of ethanol at −20°C. P^{32}-labeled RNA extracted from purified type 3 virus was heat-denatured[9] and mixed with the H^3-RNA. Electrophoresis in a 10-cm 2% polyacrylamide gel containing 0.5% agarose and 0.1% sodium dodecylsulfate was performed at 20°C and 4 ma/gel for 1¾ hr. The gel was sliced and dissolved for counting as described previously.[10] Virus RNA-P^{32} (●); H^3-labeled reaction product (○).

stranded RNA was prepared by exposing type 3 virus-infected, actinomycin-treated L cells to H^3-uridine from seven to eight hours after infection; the RNA was extracted with phenol and the radioactive single-stranded RNA was separated by methylated albumin-kieselguhr column chromatography.[9] As expected for single-stranded RNA and in contrast to viral adenine-rich ribopolymer, exposure to pancreatic RNase degraded 90 per cent or more of the radioactivity to acid-soluble material in each case (Table 2). However, both the enzymatically synthesized RNA and the single-stranded, virus-induced RNA from infected cells formed RNase-resistant duplexes when they were annealed with denatured virus RNA (Table 2). It is also likely that the *in vitro* product was copied from the double-stranded genome rather than from the single-stranded adenine-rich ribopolymer since a product complementary to *A* would contain more UMP than was available in the induction mixture. For example, the products from the complete reaction mixtures in Table 1 contained 15.5 mμmoles of CMP and 17.1 mμmoles of GMP (70.5 and 77.8 mμmoles/mg protein × 0.22 mg virus protein per reaction). If the products were complementary to *A* which

TABLE 2. *Annealing of single-stranded, virus-specific RNA with reovirus RNA.*

Treatment	RNA from Infected Cells		RNA from Reaction Product	
	Acid-precipitable cpm	Per cent RNase-resistant	Acid-precipitable cpm	Per cent RNase-resistant
None	3574	—	7620	—
Annealed + RNase	3031	85	5820	76
RNase	353	10	527	7

H^3-labeled RNA was mixed with 27 μg heat-denatured type 3 reovirus RNA in 0.5 ml 0.01 *M* phosphate buffer, pH 7, containing 0.3 *M* NaCl. Each mixture was divided into three aliquots, one of which was annealed by being slowly cooled from 90°C to room temperature.[9] The annealed sample and a second aliquot were incubated with 2 μg/ml of pancreatic RNase at 37°C for 30 min. All samples were then precipitated with 5% TCA at 4°C, collected on filters, and counted.

consists of 90 times more AMP than CMP or GMP,[18] they would also contain 1.40 and 1.54 μmoles of UMP, respectively. However, only 0.5 μmole of the nucleoside triphosphate was present in the reaction mixture.

Conclusions.—During the first stages of reovirus replication, the infecting particles are stripped of their outer layer of capsomeres by lysosomal enzymes.[6] This process has been simulated *in vitro* by treating reoviruses with chymotrypsin.[17] A rise in reovirus infectivity was observed after proteolytic digestion of capsomeres[17] probably due to activation of the virion-associated RNA polymerase. The enzyme apparently is an inner structural component of reoviruses since it becomes active after removal of the outer protein layer and remains functional in the presence of proteolytic enzymes. It may correspond to one of several virus structural proteins.[21]

On the basis of its sensitivity to RNase and content of GMP and CMP, the *in vitro* product probably does not correspond to the viral adenine-rich ribopolymer or its complement. The results of the annealing experiments indicate that most of the product is copied from viral double-stranded RNA. It is not known whether all or a limited number of the reovirus genome segments are transcribed *in vitro*. The small size of the reaction product may result from copying of short sequences in the template or from degradation during incubation.

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The early steps in the replicative cycle of viruses which contain double-stranded nucleic acid have been studied extensively. In most instances the enzymes which synthesize the first virus messenger RNA have not been identified. Although the parental genome of a DNA-containing virus presumably can be transcribed by the cellular DNA-dependent RNA polymerase, cellular enzymes which catalyze the formation of single-stranded RNA from a double-stranded RNA template like the reovirus genome have not been found. It remains to be established that the reovirus-associated RNA polymerase, like the poxvirus-bound enzyme,[13] is responsible for the synthesis of early virus messenger RNA.

The electron micrographs were kindly provided by M. D. Hoggan of this Institute.

Note added in proof: It was reported recently (Watanabe, Y., S. Millward, and A. F. Graham, *J. Mol. Biol.*, **36**, 107 (1967)) that certain segments of the parental reovirus genome are transcribed in infected cells when synthesis is inhibited by cycloheximide, and it was suggested that a polymerase carried by the virion may be responsible for this early messenger RNA synthesis.

* Present address: The Salk Institute for Biological Studies, San Diego, California. Visiting Investigator from The Roche Institute of Molecular Biology, Nutley, New Jersey.

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