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Ribonucleic Acid Polymerase Catalyzing Synthesis of Double-stranded Arbovirus Ribonucleic Acid

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The large-particle fraction from the cytoplasm of chick embryo fibroblasts infected with Semliki Forest virus was found to catalyze the incorporation of the 5'-triphosphates of guanosine, adenine, cytidine, and uridine into an acid-insoluble alkali-labile product. The conditions affecting the preparation and assay of this enzyme were investigated. The ribonucleic acid (RNA) polymerase was not present in uninfected cells, and it appeared in infected cells at the time of rapid viral RNA synthesis. The polymerase was found to catalyze the synthesis of a species of RNA which was resistant to ribonuclease and which exhibited the sedimentation properties, buoyant density, and thermal transition temperature of the double-stranded RNA found in vivo in chick cells infected with Semliki forest virus. Attempts to demonstrate that the reaction product of this enzyme also included single-stranded viral RNA were not successful. Although other interpretations are possible, these results give some support to the suggestion that more than one enzyme may be involved in the replication of viral RNA.

The replication of the nucleic acid component of many small ribonucleic acid (RNA)-containing viruses has been shown to be mediated by an RNA polymerase (RNA nucleotidyl transferase, EC 2.7.7.6) similar to the enzyme first described by Baltimore and Franklin (5, 6). Studies on polymerases isolated from cells infected with picornaviruses (3, 11), turnip yellow mosaic virus (1), and RNA bacteriophages (27) have shown that these enzymes catalyze the incorporation of nucleoside triphosphates into two species of RNA, a progeny viral RNA and a double-stranded form of RNA composed of one strand each of viral RNA and complementary RNA. The synthesis of both species of RNA was unaffected by actinomycin D or deoxyribonuclease, thus indicating that deoxyribonucleic acid DNA plays no part in the replicative process. It has been suggested (10, 12, 27) that replication of viral RNA involves two polymerases, one utilizing parental viral RNA as a template for the synthesis of a double-stranded intermediate and a second enzyme which uses this intermediate as a template for the asymmetric synthesis of progeny RNA; evidence suggesting the existence of two polymerases has been provided by the studies of Lodish and Zinder (20) on temperature-sensitive mutants of f2 bacteriophage.

Semliki forest virus (SFV), a group A arthropod-borne virus, differs from the picornaviruses in that it contains lipid and has the shape of a nonrigid sphere of approximately 50 mμ in diameter (9). It is closely related to Sindbis virus, which has been shown to consist of an RNA-protein core 30 mμ in diameter surrounded by a phospholipid membrane of cellular origin (22, 23). However, like picornaviruses, its growth is not inhibited by actinomycin (26), and presumably DNA does not participate in its replication. This report describes the isolation of an RNA polymerase from chick fibroblasts infected with this virus. It was found to catalyze the synthesis of a species of RNA which is probably identical with the double-stranded RNA present in vivo in infected cells.

MATERIALS AND METHODS

Growth and assay of virus. The origin of the Kumba strain of SFV and the chick embryo fibroblasts used in this work and the growth of the virus in monolayer cultures has already been described (E. Mécs et al., J. Gen. Virol., *in press*). For the routine preparation of polymerase, monolayer cultures in 20-oz flat bottles, containing about 2×10^8 cells in 32 ml of Gey's medium plus 0.25% lactalbumin hydrolysate, 2.5% calf serum, 0.005 M tris(hydroxymethyl)aminomethane(Tris) chloride (*p*H 7.6), and 2 μg/ml of actinomycin D, were infected with SFV at a multiplicity of 50 to 100 plaque-forming units (PFU) per cell. Cultures were kept overnight at 4 C and then warmed to 37 C; this was taken as zero-time. Overnight preincubation at 4 C was used because it was convenient; previous work in this laboratory had shown that this treatment did not affect virus growth

rate or the final yield after one cycle of replication (14). In experiments in which virus growth was followed, monolayers on 6-cm petri plates were used, and two plates per time point were frozen, thawed, and assayed for infectious virus as described previously (Mécs et al., J. Gen. Virol., *in press*).

Preparation and assay of polymerase. Infected cells were usually harvested 6 hr after infection, and the medium was decanted. The cells were washed twice with ice-cold 0.15 M NaCl and once rapidly with 0.001 M Tris chloride (*p*H 8.3), 0.01 M 2-mercaptoethanol, and 0.001 M $MgCl_2$. All ensuing operations were carried out at 0 to 2 C. The cells were scraped off with 5 ml per bottle of the hypotonic Tris-mercaptoethanol-$MgCl_2$ medium and disrupted with about 20 strokes of a Dounce homogenizer; the degree of disruption was checked by microscopic examination. Concentrated solutions of Tris chloride (*p*H 8.3), mercaptoethanol, and $MgCl_2$ were added to give final concentrations of 0.08 M, 0.01 M, and 0.004 M, respectively. The cell homogenate was diluted with an equal volume of 0.01 M mercaptoethanol; nuclei were removed by centrifugation at 600 × *g* for 10 min. The supernatant fluid was centrifuged at 10,000 × *g* for 20 min, and the surface of the pellet was rinsed with 0.04 M Tris chloride, 0.01 M mercaptoethanol, and 0.002 M $MgCl_2$. It was suspended in a small volume of the rinsing fluid and stored at −70 C. Protein was estimated by digestion and nesslerization (19) by use of a protein to total nitrogen ratio of 6.25. The polymerase preparations contained 6 to 10 mg of protein per ml.

For the assay of polymerase activity, 0.05 or 0.1 ml of the suspension (0.2 to 0.6 mg of protein) was incubated for 15 min at 37 C with the following constituents in a final volume of 0.35 ml: [^{3}H]guanosine 5′-triphosphate (GTP; specific activity = 25 c per mole), 50 mμmoles; the 5′-triphosphates of adenine, cytidine, and uridine (ATP, CTP, and UTP, respectively), 50 mμmoles of each; phosphoenolpyruvate (PEP), 5 μmoles; pyruvate kinase, 10 μg; $MgCl_2$, 0.8 μmole; actinomycin D, 1 μg; Tris chloride (*p*H 8.6), 35 μmoles; 2-mercaptoethanol, 7 μmoles. In some instances the labeled precursors used were [^{14}C]ATP (specific activity 15 c per mole), [^{14}C]UTP (25 c per mole), or [^{14}C]CTP (25 c per mole). The reaction was stopped by adding 1 ml of ice-cold 0.5 N $HClO_4$ containing 0.125 M $Na_4P_2O_7$ and then chilling to 0 C; when the amount of protein in the reaction mixture was less than 0.5 mg, 0.1 ml of carrier (5 mg of yeast RNA per ml plus 5 mg of bovine serum albumin per ml) was added before the addition of acid. The precipitate was centrifuged off and dissolved at 0 C in 0.2 ml of 1 N NaOH plus 0.8 ml of 0.125 M $Na_4P_2O_7$ and then was immediately precipitated with 2.0 ml of 0.5 N $HClO_4$. The precipitate was centrifuged and treated a second time with NaOH and $Na_4P_2O_7$. After acid precipitation and centrifugation, it was washed with 4 ml of 5% trichloroacetic acid and then was suspended in 0.5 ml of the same. The suspension was heated at 90 C for 20 min, cooled, and dissolved in 10 ml of dioxane scintillator [8.0 g of diphenyloxazole, 0.1 g of 1,4-bis-2-(4-methyl-5′-phenyloxazolyl) benzene, and 180 g of naphthalene per liter of dioxane]; both precipitate and supernatant fluid are soluble in this fluid. Radioactivity was determined in a Tri-Carb scintillation counter (Packard Instrument Co., Inc., Downers, Grove, Ill.) with efficiencies of 22% for ^{3}H and 70% for ^{14}C. All assays were corrected for incorporation into identical samples kept in an ice bath for 15 min.

In early experiments, 2-mercaptoethanol was not present in either the preparation or the assay of the polymerase; this is indicated in the text.

Extraction of RNA from enzyme reaction products and infected-cell cytoplasm. When the product of the polymerase reaction was to be studied, the specific activity of the [^{3}H]GTP was increased to 100 c per mole, the amounts of reactants were increased three- to fourfold, and the reaction was stopped by freezing in a Dry Ice-ethyl alcohol bath. The following were added per milliliter of reaction mixture: bentonite (20 mg/ml), 0.1 ml; 0.25 M sodium ethylenediaminetetraacetate (EDTA; *p*H 7.5), 0.15 ml; 0.5 M potassium phosphate buffer (*p*H 7.5), 0.15 ml. The mixture was then warmed rapidly to 37 C, and 1 ml of sodium dodecyl sulfate (SDS; 10%, w/v), previously warmed to 37 C, was added and the mixture was stirred for 4 min at 37 C. It was made 0.1 M with respect to KCl and rapidly cooled to 0 C; the precipitated potassium dodecyl sulfate was removed by centrifugation at 1,000 × *g* for 20 min at 0 C. The supernatant fluid was stored at −70 C before use. Extracts were also prepared by the SDS-phenol technique. After incubation at 37 C for 4 min with SDS and after addition of KCl to a final concentration of 0.1 M as before, an equal volume of water-saturated phenol was added. Stirring was continued for another 3 min at 37 C. The aqueous phase was separated by centrifugation and re-extracted with 0.5 volumes of water-saturated phenol. The phenol was removed from the aqueous phase with ether, and the ether was removed by a stream of nitrogen. Bentonite (1 mg/ml) was added and the extract was stored at −70 C. The ability of both methods to extract RNA from polymerase incubation mixtures was compared. The yields of radioactivity were similar with both methods, and the sucrose density gradient patterns did not differ significantly (for example, compare Fig. 9 and 11). The SDS method was used for most studies on the polymerase product, as it was faster and more convenient than the SDS-phenol method.

The two methods were also compared by use of cytoplasm from infected cells which had been previously incubated with [^{3}H] adenosine or mixtures of ^{14}C-labeled amino acids. In this instance, the SDS-phenol method extracted about 60% more RNA than when SDS alone was used, although the sucrose gradient profiles of the two extracts were very similar. For this reason, the SDS-phenol method was used for extracting RNA from cellular preparations. Extracts of infected cytoplasm made by the two methods were also compared for their protein content. As judged by their ultraviolet spectrum, the SDS extracts contained 5 to 10% of protein, whereas none could be detected in the SDS-phenol extracts. When cytoplasm from cells preincubated with labeled amino acids was extracted and examined on sucrose

gradients, less than 0.2% of the total trichloroacetic acid-insoluble radioactivity was present in the SDS extract, and all of this radioactivity sedimented in the 2 to 4S region of the gradient. Negligible radioactivity was detected in extracts made by the SDS-phenol method.

Analysis of RNA on sucrose gradients. For sucrose gradient analysis, the RNA extract (0.3 ml) was mixed with 250 μg of RNA prepared by phenol extraction of chicken liver and was layered onto a 4.4-ml linear sucrose gradient (5 to 20% sucrose; 0.1 M KCl; 0.01 M Tris chloride, *p*H 7.5; 0.001 M sodium EDTA). Gradients were centrifuged at 4 C for 2.5 hr at 38,000 rev/min in the swinging bucket rotor of the MSE model 50 ultracentrifuge. Fractions (0.22 ml) were collected by piercing the bottom of the tube, and portions (0.05 ml) were diluted for measurement of optical density at 260 mμ. The remainder was pipetted onto 2 × 4 cm strips of Whatman no. 1 filter paper and dried, and the acid-insoluble radioactivity was determined by the method described by Dalgarno et al. (11). In some instances, fractions were incubated with 2 μg of ribonuclease per ml at 30 C for 10 min before working up.

Determination of thermal transition temperature of ribonuclease-resistant RNA. SDS extracts of the polymerase reaction product were dialyzed for 24 hr at 4 C against 0.15 M NaCl and 0.015 M sodium citrate (SSC), and bentonite was removed by centrifugation at 13,000 × *g* for 30 min. Portions (0.2 ml) were sealed in 8-mm glass tubes and heated in a glycerol-water bath at the appropriate temperature for 8 min; they were then plunged into a Dry Ice-ethyl alcohol bath. The samples were thawed and incubated at 25 C for 30 min with 5 μg of ribonuclease per ml. They were cooled to 0 C, 1 ml of 0.5 N $HClO_4$ was added, and the samples were filtered through 3-cm diameter standard Oxoid membranes (Oxo Ltd., London, England). These were washed three times with ice-cold 0.25 N $HClO_4$, twice with 5% trichloroacetic acid, twice with 1:1 ethyl alcohol-ether, once with ether, and then were finally dried. The membranes were covered with scintillator (5.0 g of diphenyloxazole and 0.5 g of 1,4-bis-2-[4-methyl-5′-phenyloxazolyl]benzene per liter of toluene) and counted in a Tri-Carb scintillation counter, with an efficiency of 22% for ^3H. This method for the estimation of acid-insoluble radioactivity was also used to determine the sensitivity of various RNA preparations to ribonuclease digestion.

Other methods. Nearest-neighbor-frequency analysis of the product formed after incubation of the polymerase with [α-^{32}P]-labeled ATP was carried out as described by Dalgarno et al. (11) by use of the method of Katz and Comb (17*a*) for the separation of nucleotides. Rates of actinomycin-resistant RNA synthesis were measured by incubation of infected cells with [^3H]adenosine for 15 min at 37 C, as described previously (Mécs et al., J. Gen. Viol., *in press*). The buoyant density of the reaction product in cesium sulfate was measured as described by Dalgarno et al. (11), except that centrifugation was carried out at 35 to 40 C. Centrifugation at 15 C resulted in a variable loss of single-stranded RNA; the cause of this loss was not investigated. RNA was estimated with orcinol by the method of Hurlbert et al. (17).

Materials. [^3H]GTP, [^{14}C]ATP, [^{14}C]CTP, and [^{14}C]UTP were supplied by Schwarz Bio Research Inc., Orangeburg, N.Y., and were diluted with carrier to the specific activities indicated. [α-^{32}P]ATP was purchased from International Chemical & Nuclear Corp., City of Industry, Calif. The ATP, UTP, CTP, and GTP were commercial preparations (Sigma Chemical Co., St. Louis, Mo.). Pyruvate kinase, pancreatic ribonuclease, and the Ba-Ag salt of PEP were supplied by Boehringer & Soehne GmbH, Mannheim, Germany; the PEP was converted to the sodium salt by treatment with H_2SO_4 and HCl, and this was neutralized to a *p*H of 8.0 with NaOH. Actinomycin D was a gift of H. D. Brown of Merck & Co., Inc., Rahway, N.J. Deoxyribonuclease (electrophoretically pure and free from ribonuclease) was supplied by Worthington Biochemical Corp., Freehold, N.J. The SDS was purchased from Matheson, Coleman & Bell, Inc., Cincinnati, Ohio; before use it was washed with ethyl alcohol and ether at 0 C to remove ultraviolet-absorbing materials. SFV, grown in the presence of $^{32}PO_4$, was kindly provided by E. Mécs; it had been purified by a modification of the method of Cheng (9). ^{32}P-labeled SFV RNA was prepared from it by phenol-SDS extraction (Mécs et al., J. Gen. Virol., *in press*).

RESULTS

Subcellular localization of viral RNA synthesis and polymerase activity. Previous studies (Sonnabend, Martin, and Mécs, Nature, *in press*) had shown that most of the radioactivity from [^3H]adenosine was incorporated into three species of virus-specific RNA when infected cells were incubated for brief periods with the precursor; one of these species was resistant to digestion with ribonuclease. We felt that the enzymes responsible for viral RNA synthesis would probably occur at the same site as the synthesis of virus-specific RNA, particularly of the ribonuclease-resistant form, as this may be a possible intermediate in the replication of viral RNA. We therefore examined cell homogenates for the distribution of both ribonuclease-resistant and -sensitive newly synthesized RNA. In addition, the fractions were assayed for ability to incorporate [^{14}C]ATP into acid-insoluble material in the assay system described by Horton, Liu, Martin, and Work (16); later this was shown to be suboptimal for both the preparation and assay of SFV RNA polymerase (see below), but the estimate gave a useful confirmation of the localization of viral RNA synthesis.

At 4 hr after infection in the presence of actinomycin, cells were incubated with [^3H]adenosine (10 μc per 10^7 cells) for 15 min. At this time, synthesis of viral RNA had begun but had not yet reached a maximal rate (Mécs et al., J. Gen.

TABLE 1. *Distribution of polymerase activity, virus-specific RNA synthesis, and virus among cytoplasmic fractions of SFV-infected chick cells*

Cell fraction sedimenting after centrifugation at[a]	Percentage of total virus[b]	Percentage of ^{3}H-adenosine incorporation[b]		Percentage of ^{3}H-adenosine incorporation resistant to ribonuclease in each fraction	Percentage of total RNA polymerase activity[b]
		Sensitive to ribonuclease	Resistant to ribonuclease		
600 × *g* for 10 min	1.5	5.8	4.9	37.5	—
13,000 × *g* for 20 min	22.5	46.8	60.2	52.7	72.8
105,000 × *g* for 25 min	64.4	26.6	17.3	30.2	12.8
105,000 × *g* for 60 min	11.4	14.8	14.1	39.5	8.0
105,000 × *g* for 800 min	0.2	6.0	3.5	27.9	6.4
Total values	8.6×10^9 PFU	3.55×10^5 counts per min	2.46×10^5 counts per min		770 μμmoles of ATP incorporated

[a] The supernatant fluid from one centrifugation was used for the next. The nuclear pellet (600 × *g* for 10 min) was washed by sedimentation through 2 M sucrose at 100,000 × *g* for 30 min before examination.

[b] Total estimates for each particular fraction are expressed as a percentage of the sum totals for all fractions. The actual values for the sum of totals are given in the bottom line. All estimates were corrected for the amount of single-stranded RNA (about 10.5%) which was resistant to ribonuclease digestion under the conditions used (see text).

Virol., *in press*). The cells were washed and disrupted with a Dounce homogenizer; the homogenate was subjected to centrifugation at various speeds and for various times as indicated above, with the supernatant fluid from one centrifugation being used for the next. The pellets were suspended in 0.1 M potassium phosphate (*p*H 7.2) containing 1 mg of bentonite per ml. Portions were assayed for infectious virus, polymerase activity, and for total [^{3}H] radioactivity. The ratio of nuclease-sensitive to nuclease-resistant radioactivity was estimated by extracting the pellets with SDS, centrifuging at 15,000 × *g* for 30 min to remove bentonite, and incubating portions of the extract, made 0.15 M with respect to NaCl, with 3 μg of ribonuclease per ml at 30 C for 20 min. ^{14}C-labeled chick ribosomal RNA was added to the extracts to check the extent of hydrolysis achieved by the ribonuclease. In all cases, 89 to 90% of the ^{14}C radioactivity was rendered acid-soluble and the estimates of ribonuclease-resistant RNA were corrected for this residual resistant material. The results (Table 1) show that the greatest concentration of both nuclease-sensitive and -resistant RNA occurred in the cytoplasmic fraction sedimenting at 13,000 × *g* for 20 min. This fraction also contained practically all of the polymerase activity. The proportion of ribonuclease-resistant RNA in this fraction (52.7%) was higher than in any other. This large-particle cytoplasmic fraction was therefore used as a source of polymerase in all subsequent studies. The high proportion of the total newly synthesized RNA which was resistant to ribonuclease (41%) was due to the brief labeling period used. With a 15-min pulse, incorporation occurs almost entirely into a mixture of nuclease-resistant and -sensitive RNA in approximately equal proportions (15; J. A. Sonnabend et al., Nature, *in press*). Infectious virus was located in the fraction expected for a particle of its size [100,000 × *g* for 25 min; Cheng (9)]. The nuclear fraction was sedimented through 2.2 M sucrose to free it of cytoplasm and examined separately for its ability to incorporate [^{14}C]ATP. Moderate levels of activity were found in the uninfected nuclei, but this activity was 50% less in nuclei from infected cells. The activity increased slightly when the three nucleoside triphosphates were omitted, and hence it was not considered to be relevant to the synthesis of viral RNA. These observations suggest that the synthesis of SFV viral RNA takes place in the large-particle fraction of the cytoplasm, a situation similar to that found in cells infected with poliovirus (4), mengovirus (5), and encephalomyocarditis virus (10).

Polymerase assay conditions. When the large-particle cytoplasmic fraction from SFV-infected chick cells was incubated with [^{3}H]GTP under the assay conditions given in the Materials and Methods section, radioactivity was rapidly incorporated into an acid-insoluble product for the first 15 min; thereafter the incorporation rate declined (Fig. 1). The rate of incorporation was

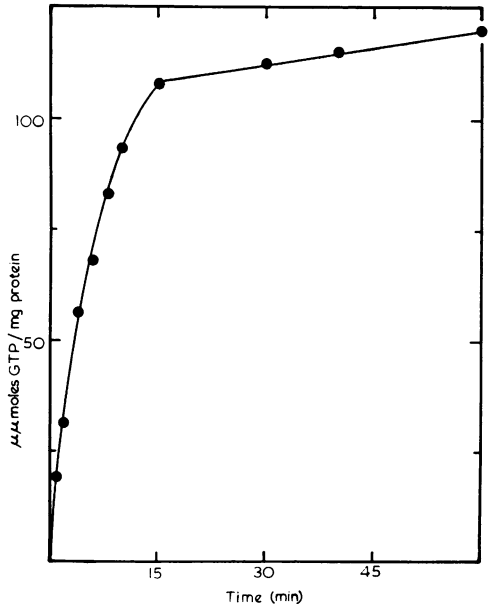

FIG. 1. *Time course of [³H]GTP incorporation. Polymerase was prepared from chick cells 5.5 hr after infection with SFV. It was incubated at 37 C for various times with [³H]GTP, ATP, CTP, UTP, PEP, phosphokinase, $MgCl_2$, actinomycin D, 2-mercaptoethanol, and Tris buffer (pH 8.6) in a total volume of 0.35 ml, and the acid-insoluble radioactivity was measured as described in Materials and Methods.*

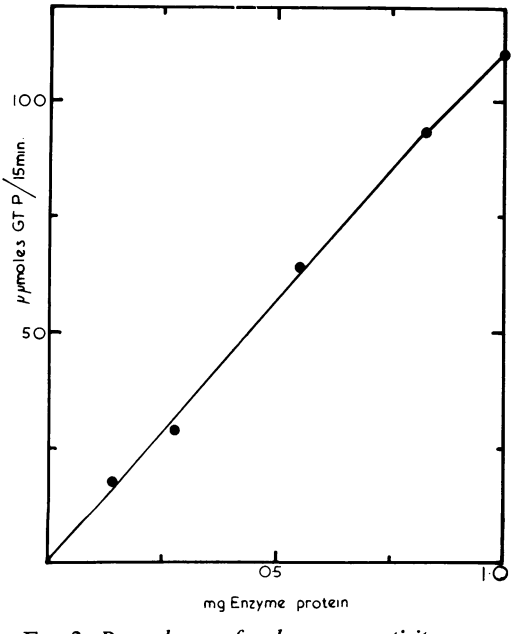

FIG. 2. *Dependence of polymerase activity on enzyme concentration. Various quantities of polymerase from 6 hr-infected cells were incubated as described in the legend to Fig. 1.*

directly proportional to the amount of polymerase added (Fig. 2).

The *p*H optimum for incorporation was 8.0 to 8.3, as shown in Fig. 3. Tris chloride buffer was preferable to Tris maleate, but it was necessary to use a buffer of higher *p*H than the optimum (e.g., 8.6 to 8.8) to overcome the buffering effect of the high concentrations of polyphosphates in the assay mixture. Magnesium ions were necessary for maximal activity, with an optimal concentration of 0.8 μmole per assay (Fig. 4). Manganese could not replace magnesium (Fig. 4), and when part of the magnesium was replaced with manganese it was inhibitory.

At low concentrations of nucleoside triphosphates (5 to 30 mμmoles/mg of enzyme protein), incorporation of [³H]GTP was proportional to the substrate concentration (Fig. 5), but saturation was achieved at about 100 mμmoles. PEP was not essential, but it was required for maximal activity (Fig. 5). High salt concentrations were inhibitory; e.g., NaCl added to final concentrations of 0.5 M, 1.0 M, and 2.5 M resulted in polymerase activities which were 52, 32, and 9%, respectively, of the control, whereas 1 M

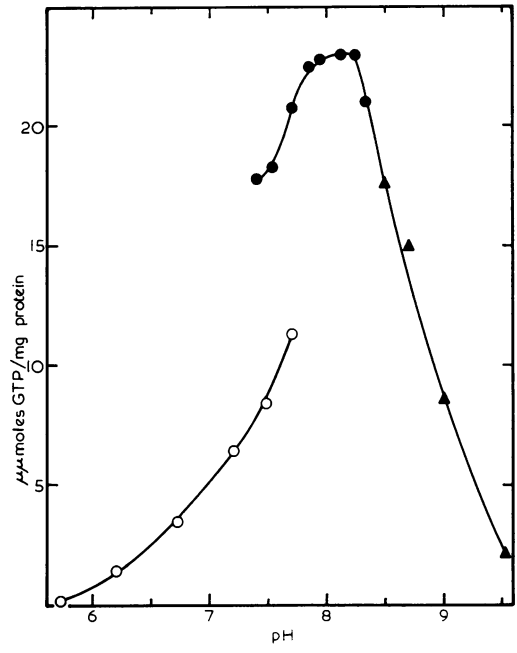

FIG. 3. *Effect of pH on SFV polymerase activity. Polymerase from 5.25 hr-infected cells was incubated as described in the legend to Fig. 1 except for the buffer pH. In each case, the Tris concentration was 35 μmoles per assay. The pH indicated was that of the incubation mixture and not of the buffer used. (○) Tris maleate buffer; (●) Tris chloride buffer; (▲) Tris-NaOH.*

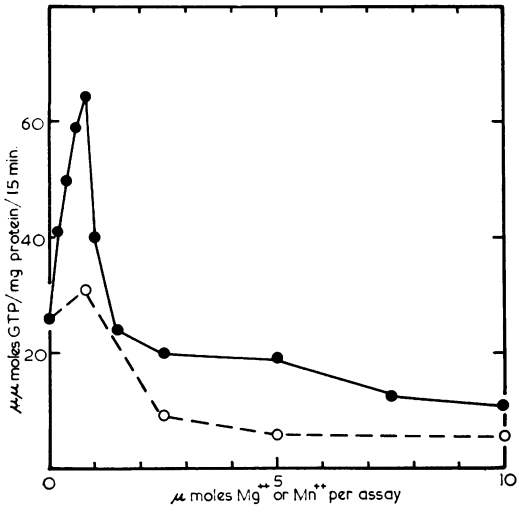

FIG. 4. *Effect of Mg^{++} and Mn^{++} concentration on polymerase activity. The assay conditions were those described in the legend to Fig. 1. Values for divalent ion concentration are given as micromoles per assay.* (●) *$MgCl_2$;* (○) *$MnCl_2$.*

ammonium sulfate caused an 89% inhibition of activity.

Polymerase preparations from infected cells incorporated labeled ATP, GTP, CTP, and UTP into an acid-insoluble product, and the presence of the other three unlabeled nucleoside triphosphates was essential for maximal incorporation (Table 2). CTP was incorporated to a much greater extent than the other three precursors, but only about one-third of this activity was dependent on the presence of all four nucleoside triphosphates. This may be due to the presence of a CTP polymerase similar to that described by Edmonds (13). The incorporation of ribonucleoside 5′-triphosphates by "polymerase" preparations from uninfected cells was, in general, far less than that observed with infected preparations, and, when labeled ATP and CTP were used, it was stimulated by the omission of the other three unlabeled triphosphates. The high levels of ATP incorporation in the absence of other triphosphates by both infected and uninfected preparations suggested that enzymes catalyzing incorporation of ATP into a nonviral product were present. Such enzymes, e.g., those catalyzing the synthesis of polyadenylic acid or those which add adenylic acid residues terminally to transfer RNA, have been reported in a variety of uninfected animal or bacterial cells (2, 6, 8, 16, 18, 28). This type of enzymatic activity, when found in the cytoplasm of mammalian cells, is not affected by deoxyribonuclease or actinomycin D, and it is partially inhibited by the addition of other ribonucleoside 5′-triphosphates (16, 28). It is likely that a similar enzyme was responsible for the incorporation of ATP (in the absence of other nucleoside 5′-triphosphates) in the large-particle preparations from both infected and uninfected cells. This was also suggested by the results of experiments in which chick ribosomal RNA was added to the assay; for example, 100 μg of RNA added to a complete assay of a polymerase prepared from infected cells increased the incorporation of [^{14}C]ATP from 40 to 140 μμmoles per 15 min per mg of protein, and the same quantity of RNA stimulated incorporation of ATP by uninfected preparations from 38 to 450 μμmoles per 15 min per mg of protein. On the other hand, incorporation of GTP showed a marked dependence on the presence of ATP, CTP, and UTP (Table 2) and was not affected by the addition of exogenous RNA. This indicated the absence of any polyguanylic synthetase activity, and, for this reason, [^{3}H]GTP was routinely used in the viral polymerase assay.

The incorporation of [^{3}H]GTP by the viral polymerase was not affected by the addition of up to 25 μg of deoxyribonuclease to the assay. Pancreatic ribonuclease was partially inhibitory; e.g., 1 μg added to the assay caused a 15% decrease in incorporation, and 12.5 μg resulted in a 36% inhibition. The activity of the enzyme is stimulated by the presence of 2-mercaptoethanol

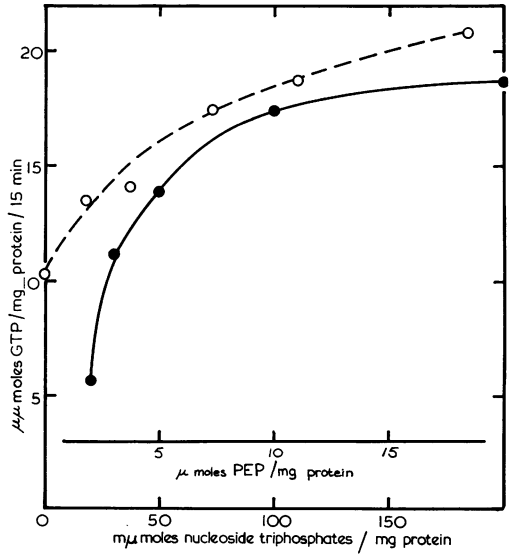

FIG. 5. *Effect of nucleoside triphosphate and PEP concentrations on SFV polymerase activity. The assay conditions were those described in the legend to Fig. 1. [^{3}H]GTP, ATP, CTP, and UTP were added at the concentrations indicated.* (●) *Effect of nucleoside 5′-triphosphates;* (○) *effect of PEP.*

TABLE 2. *Incorporation of various ribonucleoside 5'-triphosphates by enzymes from uninfected and infected cells*

Labeled substrate	Source of polymerase	Polymerase activity[a] when three other nucleoside triphosphates were	
		Present	Absent
	cells		
[^{14}C]ATP	Infected	100	53.5
	Uninfected	18.0	46.0
[^{14}C]UTP	Infected	39.6	14.3
	Uninfected	12.4	5.7
[^{3}H]GTP	Infected	73.8	9.9
	Uninfected	7.3	10.7
[^{14}C]CTP	Infected	219	146
	Uninfected	32.6	46.6

[a] Expressed in micromicromoles per milligram of protein.

TABLE 3. *Effect of 2-mercaptoethanol on the preparation and assay of viral RNA polymerase*

Mercaptoethanol added		Polymerase activity (μμmoles of GTP per mg of protein)
During preparation[a]	In assay	
M	*μmoles*	
0	0	19.5
	1.4	35.1
	2.8	45.2
	7.0	49.1
0.05	0	39.3
	1.4	50.2
	2.8	54.8
	7.0	56.3

[a] Mercaptoethanol was present at the concentration indicated in all reagents used during and after the disruption of the cells and in the medium used to suspend the enzyme.

in the assay; mercaptoethanol also assists in preserving the activity during the preparation of the polymerase (Table 3). When it was added during both the preparation and assay of the polymerase, the activity increased nearly threefold.

Time course of appearance of polymerase during virus infection. The large-particle fraction was prepared from cells at various times after infection and was assayed for polymerase activity and virus content. At the same time, replicate cultures were incubated with [^{3}H]adenosine for 15 min to measure the rate of synthesis of virus-specific RNA (2 μg of actinomycin per ml was present in all cultures). The results in Fig. 6 show that polymerase activity begins to appear at 3 hr after infection, at about the same time as the increase in viral RNA synthesis, but well before the appearance of mature virus. The rise in RNA synthetic rate is erratic, with subsidiary peaks of synthetic activity appearing before the maximal rate at 6 hr postinfection.This phenomenon in chick fibroblasts infected with SFV has been described previously (26; Mécs et al., J. Gen. Virol., *in press*). It is accompanied by changes in the proportions of the three virus-specific species of RNA (see below), and its cause is at present under investigation. The polymerase activity does not reflect these oscillations in virus RNA synthetic activity.

Nearest-neighbor-frequency analysis of the polymerase product. Viral RNA polymerase was incubated with [α-^{32}P]ATP in the standard assay system, and the RNA product was extracted with SDS, precipitated with perchloric acid, washed, and hydrolyzed with 0.3 N NaOH. This treatment rendered 93% of the radioactivity acid-soluble. The four nucleoside monophosphates were separated by column chromatography and the distribution of radioactivity among them was determined. The results of one such experiment were (mean of duplicates): adenylic acid, 31.8%;

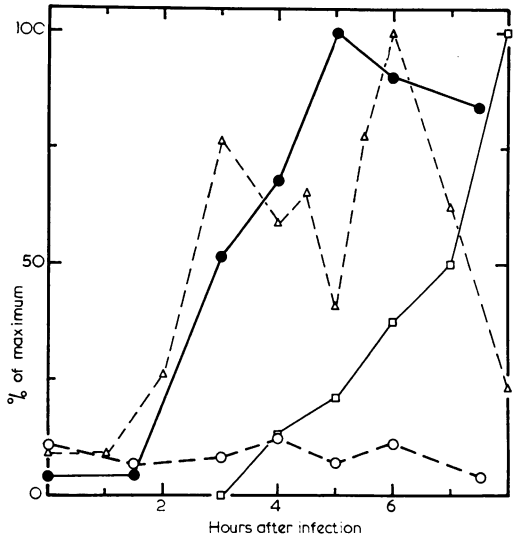

FIG. 6. *Rates of viral RNA synthesis and appearance of polymerase activity during course of infection of actinomycin-treated cells with SFV.* (●) *RNA polymerase activity, assayed as described in the legend to Fig. 1 (maximal value 23.5 μμmoles of GTP per mg of protein per 15 min);* (○) *polymerase activity assayed with [^{3}H]GTP as the sole nucleoside 5'-triphosphate;* (△) *rate of incorporation of [^{3}H]adenosine into total cell RNA (maximal value 152 counts per min per 15 min per μg of RNA);* (□) *intracellular virus titer (maximal value 3.2 × 10^{10} PFU per ml of culture fluid).*

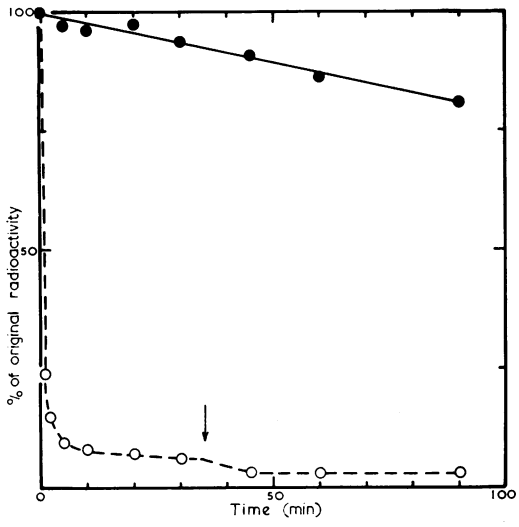

FIG. 7. *Effect of ribonuclease on the SFV polymerase reaction product and SFV RNA. RNA was extracted with SDS from a polymerase reaction mixture after incubation for 15 min with [^3H]GTP, and mixed with RNA extracted with SDS-phenol from ^{32}P-labeled purified SFV. Ribonuclease (0.5 μg/ml) was added and acid-precipitable radioactivity measured after incubation for various times at 25 C. At the time indicated by the arrow, the ribonuclease concentration was increased to 2.5 μg/ml. The results are expressed as a percentage of the ^3H and ^{32}P radioactivity present in the mixture at zero-time (1,850 counts per min per ml and 1,700 counts per min per ml, respectively). (●) Polymerase reaction product (^3H); (○) ^{32}P-labeled SFV RNA.*

cytidylic acid, 27.6%; guanylic acid, 20.9%; uridylic acid, 19.7%. These results indicate that the viral polymerase catalyzes the synthesis of RNA containing all four nucleotides in an approximately random sequence and that it is not synthesizing merely homopolymeric chains.

Resistance to ribonuclease digestion of polymerase product. The reaction product obtained after incubation of polymerase with [^{3}H]GTP was extracted with SDS and dialyzed against SSC. It was mixed with RNA from purified SFV which had been grown in the presence of $^{32}PO_4$, and Tris buffer (*p*H 7.8) was added to a final concentration of 0.05 M. It was then incubated at 25 C with 0.5 μg of ribonuclease per ml, and the loss of acid-precipitable radioactivity was measured at various times (Fig. 7). The polymerase reaction product was extremely resistant to digestion by ribonuclease, even when the concentration of ribonuclease was increased to 2.5 μg/ml; on the other hand, RNA extracted from purified virus was rapidly rendered acid-soluble.

Thermal transition temperature of resistance to ribonuclease. The pronounced resistance of the polymerase reaction product to ribonuclease suggested that the major component may be a double-stranded RNA and thus may be convertible to a single-stranded ribonuclease-sensitive form by heating; this possibility was investigated. The polymerase reaction mixture was extracted with SDS and dialyzed against SSC; portions were heated, cooled rapidly, and incubated with ribonuclease. The effect of heating on the acid-insoluble radioactivity remaining after this treatment is shown in Fig. 8. The ribonuclease resistance of the enzyme reaction product was unaffected by heating to 90 C; at higher temperatures, there was a sharp fall in ribonuclease resistance, with a transition temperature (Tm) of 103 C.

Further evidence for the identity of the polymerase reaction product comes from a comparison of its Tm value with that of the ribonuclease-resistant material found in infected cells in vivo. This latter was prepared by labeling actinomycin-treated, SFV-infected cells with

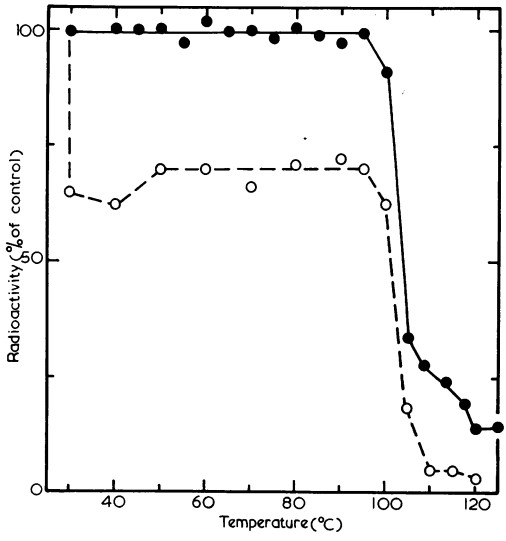

FIG. 8. *Thermal denaturation of ribonuclease-resistant RNA from SFV polymerase reaction product and of 20S RNA from labeled infected cells. RNA was extracted with SDS from a reaction mixture after incubation of polymerase with [^3H]GTP for 15 min and was dialyzed against SSC. Portions were heated at the indicated temperature and cooled. They were incubated with 5 μg/ml of ribonuclease (30 min at 25 C), and acid-precipitable radioactivity was measured (●). Material from the 20S region of a sucrose gradient run of RNA extracted with SDS-phenol from infected cells labeled for 2 hr with [^{14}C]uridine (see Fig. 9B) was diluted with SSC and the effect of temperature on ribonuclease resistance measured (○). Results are expressed as a percentage of the acid-insoluble radioactivity of unheated samples before ribonuclease treatment.*

[^{14}C]uridine from 4 to 6 hr after infection and extracting the RNA with phenol-SDS; the extract was sedimented through a sucrose gradient, and the fractions in the 20*S* region, containing ribonuclease-resistant material (see below), were pooled. The effect of heating on the ribonuclease resistance of this material was measured, and the results are given in Fig. 8. Under these same conditions, the material labeled in vivo showed a sharp transition to ribonuclease sensitivity at the same Tm value (103 C) as that of the polymerase reaction product; the fraction of this material sensitive to ribonuclease before heating (about 40%) represents contamination with ribonuclease-sensitive 26*S* (see Fig. 9 B). These results give strong support to the proposal that the enzyme reaction product and the RNA synthesized in infected cells both contain double-stranded products which are probably identical.

Sucrose gradient analysis of polymerase reaction product. Extracts of reaction products formed on incubation of the viral polymerase with [^{3}H]GTP or [α-^{32}P]ATP were examined on sucrose gradients as described in the Materials and Methods section. Only one major peak of radioactivity was seen, with an *S* value of approximately 20*S* (Fig. 9 A). A second peak at about 4*S* was seen when labeled ATP had been used in the incubation mixture, but it was diminished (Fig. 9 B) or absent (Fig. 11) when [^{3}H]GTP was used; it probably represents terminal addition to low molecular weight RNA components (11). Polymerase reaction products extracted with either SDS alone or by the phenol-SDS method showed almost identical gradient profiles.

RNA extracted from infected cells labeled in vivo exhibited two major peaks, with *S* values of 45 and 26*S* (Fig. 9 B). These peaks have been identified as progeny viral RNA (45*S*) and a mixture of 26*S* ribonuclease-sensitive RNA and 20*S* double-stranded RNA (15; Sonnabend et al., Nature, *in press*). The 20*S* RNA of the polymerase reaction product, which was resistant to digestion with ribonuclease, sedimented in the same position as the ribonuclease-resistant 20*S* RNA extracted from the infected cells labeled in vivo (Figs. 9 B and C); this suggests that these two RNA species are identical.

Buoyant density in cesium sulfate of polymerase product. When the reaction product, formed on incubation of the viral polymerase with [^{3}H]GTP, was extracted with SDS and when the RNA was centrifuged in a concentrated Cs_2SO_4 solution until equilibrium was attained, only a single peak of radioactivity was seen (Fig. 10; E. M. Martin, Proc. Meeting Federation European Biochem. Soc., 3rd, Warsaw, *in press*). This peak exhibited the same density, 1.615 to 1.625 g/cm^3, as the ribonuclease-resistant component found in actinomycin-treated infected cells labeled with [^{14}C]uridine (Martin, *in press*) and with the 20*S* material obtained from the sucrose gradient analysis of

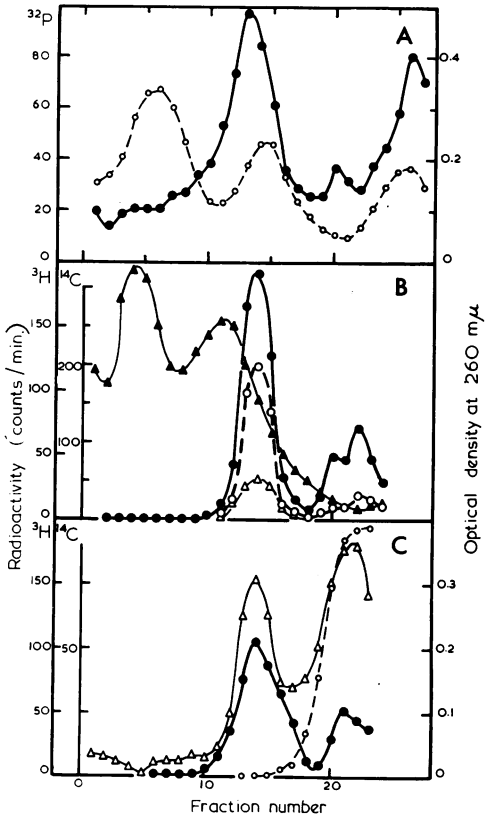

FIG. 9. *Sucrose gradient analysis of SFV polymerase reaction product. (A) Sedimentation profile of the RNA extracted with SDS from the polymerase after incubation with [α-^{32}P]ATP for 15 min. Details of extraction and gradient analysis are given in the text. (●) Acid-insoluble ^{32}P radioactivity; (○) optical density at 260 mμ of chick ribosomal RNA added as a marker. (B) RNA was extracted with SDS from the polymerase after incubation with [^{3}H]GTP. It was mixed with chick ribosomal RNA and with RNA extracted from actinomycin-treated cells which had been incubated with [^{14}C]uridine from 0 to 7 hr after infection with SFV. After centrifugation on a sucrose gradient, fractions were assayed for total and ribonuclease-resistant radioactivity. ^{3}H radioactivity of polymerase product before (●) and after (○) ribonuclease treatment; ^{14}C radioactivity of in vivo-labeled RNA before (▲) and after (△) ribonuclease treatment. (C) Procedures same as for (B) except that the mixed extracts were treated with 2 μg of ribonuclease per ml for 30 min at 25 C before centrifugation. (●) ^{3}H radioactivity; (△) ^{14}C radioactivity; (○) optical density. In all figures, fraction 1 is from the bottom of the tube.*

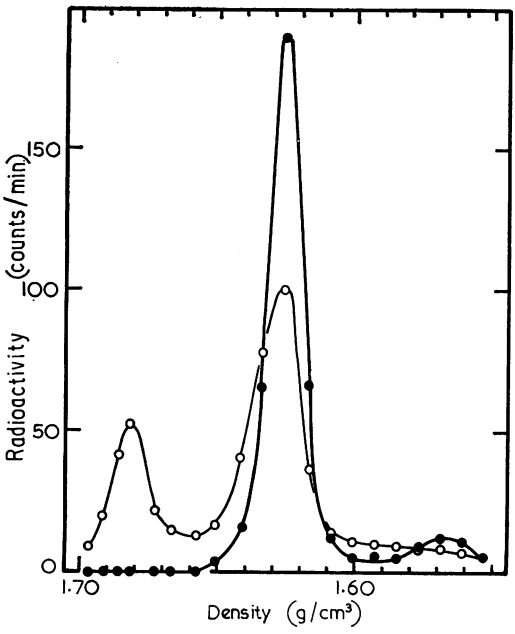

FIG. 10. *Buoyant density in cesium sulfate of SFV polymerase reaction product. RNA was extracted with SDS from the polymerase after incubation with [³H]GTP and was mixed with 20S RNA obtained by extraction with SDS-phenol of actinomycin-treated SFV-infected cells which had been labeled with [¹⁴C]uridine, followed by separation on a sucrose gradient. (●) ³H radioactivity of polymerase product; (○) ¹⁴C radioactivity of in vivo-labeled 20S RNA.*

the RNA labeled in vivo (Fig. 10). This latter preparation contained some ribonuclease-sensitive virus-specific RNA which banded at a significantly higher density (1.680 g/cm³; Fig. 10).

Attempts to detect single-stranded RNA in the enzyme reaction product. Generally, where the products formed by a virus-specific RNA polymerase have been examined, it was found that the enzyme catalyzed the synthesis of at least two species of RNA, a ribonuclease-resistant double-stranded form similar to that reported here and single-stranded progeny viral RNA (3, 11, 24, 27). Our failure to observe progeny RNA among the products of the SFV polymerase could have been due to: (i) deficiencies in our techniques for the preparation and assay of the enzyme and extraction of its products; (ii) degradation of viral RNA by ribonuclease contaminating the preparations; or (iii) the possibility that the synthesis of single-stranded viral RNA was the function of some enzymes other than the polymerase we have been examining.

The most likely possibility was that single-stranded RNA was synthesized by the polymerase, but was degraded by ribonuclease. To examine this possibility, ³²-P-labeled SFV RNA extracted from purified virus was added to the reaction mixture (supplemented with bentonite) 5 min after the start of incubation, which was then continued for another 10 min. Sucrose gradient analysis of the RNA extracted with SDS showed that about 60% of the ³²P viral RNA had been degraded to slower sedimenting components; the remaining 40% sedimented with a sharp peak in the 45*S* region, whereas there was no trace of any peak of ³H (from the [³H]GTP added to the reaction mixture) in this region. This showed that the polymerase preparations contained ribonuclease activity, which may have degraded any single-stranded RNA which might have been formed.

A further approach to the problem was to investigate the products formed in the reaction mixture after only brief periods of incubation. A typical result is illustrated in Fig. 11. RNA was extracted from reaction mixtures after incubation of polymerase with [³H]GTP for various times. In this instance, the SDS-phenol method was used for extraction, as it was possible that SDS alone either may not have extracted single-stranded RNA (although it does so from infected cell cytoplasm) or that sufficient ribonuclease may have been carried through the SDS deproteinization to have caused breakdown of single-stranded RNA (this also was unlikely as none of the labeled RNA in SDS extracts of infected cell cytoplasm or of encephalomyocarditis virus RNA polymerase products was rendered acid-soluble by incubation at 37 C for

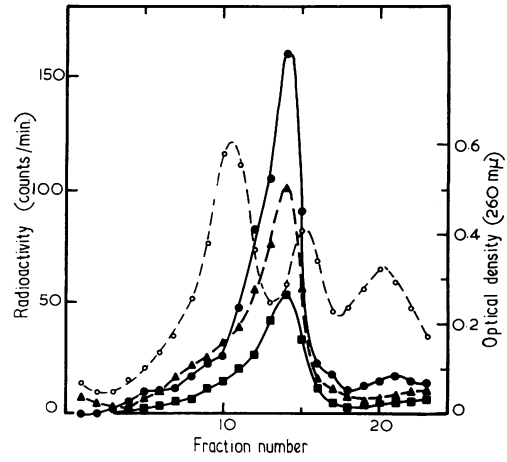

FIG. 11. *Gradient analysis of the products formed after incubation of SFV polymerase with [³H]GTP for 5 (■), 10 (▲), and 20 min (●), followed by extraction with SDS-phenol. Optical density at 260 mμ (○).*

20 min). As seen in Fig. 11, the sucrose gradient profiles of products formed after 5, 10, and 20 min of incubation were essentially the same and similar to those obtained with SDS extracts (e.g., Fig. 9); none shows evidence of any component other than the 20*S* RNA.

It was possible that synthesis of single-stranded RNA may have occurred only at certain stages during the growth of the virus. Therefore, polymerase was prepared from cells infected for for 3, 4, 5, 6, and 7.5 hr and reaction products were extracted after incubation with [^{3}H]GTP. The sucrose gradient profiles of all the products were similar and differed only in the amount of radioactivity incorporated; only a single peak at 20*S* was seen (e.g., Fig. 9A). It was thought that a higher magnesium concentration may have been required for optimal synthesis of single strands; the results in Fig. 4 suggested that this was possible. Reaction products obtained by incubation of polymerase at the optimal Mg^{++} concentration (0.8 μmole per assay) were compared with those obtained when 5 μmoles of Mg^{++} had been added, and again only a single peak of radioactivity at 20*S* was seen. Therefore, we have been forced to conclude that either our polymerase preparations contain a polymerase which only catalyzes double-stranded RNA synthesis, or that the assay conditions we have used do not permit the synthesis or detection of any single-stranded progeny RNA.

DISCUSSION

The results reported here show that the large-particle cytoplasmic fraction from chick cells infected with SFV contains an RNA polymerase whose activity is dependent on the presence of GTP, ATP, CTP, and UTP and which catalyzes the incorporation of all of them into a ribopolynucleotide. This enzymatic activity is low or absent in uninfected cells (Table 2), and, during infection, it increases at the time at which viral RNA synthesis begins (Fig. 6). Therefore, it is probable that the synthesis of this polymerase is induced by virus infection and hence may be coded for by viral RNA. This enzyme catalyzes the synthesis of a product which is resistant to digestion by ribonuclease (Fig. 7), and this resistance is abolished by heating with a sharp transition temperature of 103 C (Fig. 8). Its buoyant density in Cs_2SO_4 (about 1.62 g/cm^3) is substantially lower than that of progeny viral RNA, 1.68 (Fig. 10). It sediments in sucrose gradients with an *S* value of about 20*S* (Fig. 9). In all these respects, its properties closely resemble those of the double-stranded replicative forms of encephalomyocarditis viral RNA first described by Montagnier and Sanders (21) and have been found since among the products formed by a number of mammalian viral RNA polymerases (3, 11). When the RNA synthesized in vitro by the polymerase was directly compared with the ribonuclease-resistant RNA extracted from infected cells incubated in vivo with labeled precursors, they were found to be almost identical in a number of properties (e.g., Fig. 8–10). Therefore, we conclude that the product isolated from the SFV polymerase reaction mixture is double-stranded RNA and that it is probably identical with the ribonuclease-resistant RNA isolated in vivo in SFV-infected cells. Presumably, the polymerase is responsible for synthesis of this RNA in vivo.

Attempts to demonstrate that our polymerase preparations are capable of synthesizing single-stranded progeny viral RNA perhaps did not succeed for a number of reasons. It is probable that some single-stranded RNA, had it been formed, would have been degraded by ribonuclease contaminating our preparations. Nevertheless, the experiment in which ^{32}P-labeled SFV RNA was added to the incubation mixture suggested that some 45*S* RNA would be expected to survive, although this would depend upon the absolute amount of progeny RNA formed. The only evidence unfavorable to this explanation is the lack of any appreciable radioactivity in the 2 to 10*S* region of the sucrose gradients. Treatment of extracts containing 45*S* SFV RNA with ribonuclease resulted in the appearance of the majority of degraded RNA in this region of the gradient (e.g., Fig. 9 C). It seems unlikely that our failure to find 45*S* RNA in the reaction product is due to the extraction methods used. The SDS method is capable of extracting single-stranded viral RNA from cells infected with polio or encephalomyocarditis viruses (6*a*, 10) or from the polymerases obtained from cells infected with these viruses (3, 11). The SDS-phenol method, which gives essentially similar results as the SDS method, has been used successfully by numerous workers to extract RNA from a wide variety of viruses (25). It is possible that the double-stranded RNA formed in the reaction product is an artifact resulting from deproteinization, that the polymerase is in fact synthesizing progeny RNA, but that it is hybridizing with complementary strands present in the preparation. The formation of possible artifacts in this way during extraction of RNA from MS2 phage polymerase has been reported by Borst and Weissmann (7). This is difficult to test since all methods of deproteinization tested by Borst and Weissmann resulted in an increase in the propor-

tion of RNA resistant to ribonuclease, although at least 30% of the RNA always remained sensitive to nuclease. A similar situation may have arisen in the studies described here. However, it is unlikely to explain the complete absence of single-stranded RNA. In any case, treatment of the reaction product with ribonuclease during incubation (i.e., before extraction) caused only a 15% loss of incorporated radioactivity; if single-stranded RNA had been the major product, this treatment would have caused a much greater decrease.

It is therefore impossible to decide with any certainty whether single-stranded RNA was or was not synthesized by the SFV polymerase. If it was not formed, then it implies either that a second enzyme catalyzing single-stranded RNA synthesis, possibly using double-stranded RNA for its template as suggested by the genetic studies of Lodish and Zinder (20), must exist but has not been detected by us, or that some factor required by the polymerase, perhaps to remove the product from its template, has been lost during the preparation of the enzyme and that the type of reaction observed here in vitro is not necessarily that which normally occurs in the infected cell. This latter possibility has been suggested by Plagemann and Swim (24), who found that treatment with deoxycholate of an RNA polymerase from cells infected with mengovirus resulted in a preparation which synthesized only double-stranded RNA, whereas, before treatment, it catalyzed the synthesis of both single- and double-stranded forms of viral RNA. It is important to stress that the demonstration reported here, that infected cells contain an enzyme catalyzing the synthesis of a species of double-stranded RNA resembling that isolated from infected cells, does not permit any firm conclusions to be made as to whether or not this species of RNA plays an essential role in the synthesis of progeny viral RNA.

ACKNOWLEDGMENTS

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