INACTIVITY OF PURIFIED REOVIRUS RNA AS A TEMPLATE FOR E. COLI POLYMERASES IN VITRO

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It has been shown previously that actinomycin blocks reovirus replication in tissue culture cells.[1] Furthermore, double-stranded reovirus RNA has been reported to function like DNA *in vitro* as a template for *E. coli* RNA polymerase.[2] This function was inhibited by actinomycin, although the RNA, unlike DNA, did not appear to interact significantly with the antibiotic. In view of the observation that the replication of reovirus in cultured cells is unaffected by concentrations of actinomycin which inhibit cell RNA synthesis by 90 per cent,[3] it seemed important to investigate further the nature of the template activity of reovirus RNA. The results of this study show that purified virus RNA does not direct polynucleotide synthesis by either RNA polymerase or DNA polymerase of *E. coli*.

Experimental Procedure.—Materials: Actinomycin C_1 and X_2 were obtained from Merck and Company and Dr. E. Reich, respectively. Actinomycin C_1-H^3 (specific activity = 250 μc/μmole) and X_2-C^{14} (20 μc/μmole) were gifts of Dr. H. Weissbach and Dr. Reich. Optical grade CsCl was purchased from Harshaw Chemical Company, Cs_2SO_4 from Gallard-Schlesinger Corporation, unlabeled nucleoside triphosphates from Pabst Laboratories, and radioactive triphosphates and nucleosides from Schwarz BioResearch, Inc., and the New England Nuclear Corporation. Snake venom and calf spleen phosphodiesterases, and crystalline as well as electrophoretically purified pancreatic DNase I were purchased from Worthington Biochemical Corporation. Crystalline micrococcal nuclease was purified and provided by Dr. H. Taniuchi. RNase (Sigma Co., 5× crystallized) was heated to 80°C for 10 min before use.[4] Highly polymerized calf thymus DNA was obtained from Sigma Co., and L cell DNA was prepared according to Marmur.[4] dGdC was the gift of Dr. E. Reich. RNA polymerase, fraction IV, and DNA polymerase, fraction VII, of *E. coli* were purified according to procedures in references 5 and 6. Exonucleases I and III were purified by modified, previously described procedures.[7, 8]

Virus infection: Mouse L-929 or HeLa S3-1 cells were grown in suspension culture in Eagle's medium containing 2–5% fetal bovine serum. Reovirus type 3 stocks containing $\sim 1 \times 10^{10}$ PFU/ml were prepared and assayed as described by Gomatos *et al.*[1] For infection, 100 ml of cells (1×10^7/ml in growth medium containing 2% serum) received 10–20 PFU/cell. After 3 hr adsorption at room temperature, the suspension was diluted to 2 liters and incubated for 48 hr at 37°C. Yields in excess of 1000 PFU/cell were routinely obtained, and more than 80% of the virus was cell-associated under these conditions. The cells were collected by centrifugation and resuspended in 40 ml phosphate-buffered saline (PBS).

Purification of virus and viral RNA: The cell suspension was frozen and thawed six times, and all subsequent operations carried out at 0–4°C. After treatment with the fluorocarbon, genetron 113,[9] the virus suspension was carefully layered over 5 ml of a CsCl solution ($\rho = 1.39$), and centrifuged in the SW-25 rotor of the Spinco model L centrifuge at 24,000 rpm for 1 hr. The band of reovirus which appeared in the CsCl layer was collected, adjusted to a density of 1.38, and centrifuged at 33,000 rpm for 18–24 hr in the Spinco SW-39 rotor. The virus band was collected, and dialyzed against phosphate-buffered saline (PBS) containing 0.005 *M* $MgCl_2$. Crystalline DNase was added to the virus suspension at a final concentration of 200 μg/ml, and the mixture was incubated for 1 hr at room temperature. Following centrifugation (2 hr, 33,000 rpm, SW-39), the virus pellet was resuspended in 1 ml SSC (0.15 *M* NaCl, 0.015 *M* sodium citrate), and viral RNA was obtained by two extractions of the suspension at room temperature with an equal volume of H_2O-saturated phenol. Excess phenol was removed from the aqueous phase by ether extraction, and the RNA was precipitated at −20°C by the addition of 2 vol of ethanol, collected by centrifugation, dissolved in SSC, and frozen.

Purification of reovirus RNA in gradients of Cs_2SO_4: A solution of RNA, 10 μg/ml, in SSC containing Cs_2SO_4 (ρ = 1.60) was centrifuged at 4°C for 5 days at 33,000 rpm in the SW-39 Spinco rotor. Each tube in the rotor contained 5 ml of solution. Following centrifugation 0.2–0.3-ml fractions were collected dropwise. The optical density at 260 mμ was measured and the density of selected fractions was determined with an Abbé refractometer. The fractions containing the RNA were pooled, dialyzed against SSC, precipitated with 2 vol of ethanol, and stored frozen at a concentration of 0.2–1.0 mg/ml in 0.01 *M* tris pH 7.9, 0.01 *M* NaCl. RNA concentrations were determined by assuming an optical density at 260 mμ of 1.0 for a solution containing 50 μg/ml.

Determination of sedimentation coefficient: RNA was diluted to a concentration of 20 μg/ml in 0.01 *M* phosphate buffer, pH 6.7, containing 0.72 *M* NaCl. Centrifugation in the Spinco model E analytical centrifuge was performed at 52,640 rpm and 25°C, and photographs were taken at 4-min intervals with ultraviolet absorption optics. Sedimentation coefficients were calculated from tracings of the negatives made with an Analytrol densitometer.

Enzyme assays: RNA polymerase: The standard assay mixture was that of Chamberlin and Berg,[5] but the final volume was reduced to 0.05 ml. H^3-labeled GTP, CTP, and ATP were the radioactive substrates in different experiments. After incubation at 37°C for 15 min, the vessel was chilled and 1 ml 5% trichloroacetic acid (TCA) added. The acid-insoluble material was collected on a Millipore filter, washed three times with 1 ml 5% TCA, dried, and counted in 15 ml liquifluor-toluene in a scintillation counter.

DNA polymerase: The standard assay mixture in a final volume of 0.3 ml was that of Richardson *et al.*[6] The labeled substrate was dTTP-H^3 (8×10^3 cpm/mμmole). The vessels were incubated at 37°C for 15 min, chilled, and assayed as described in the legend to Table 4.

Results.—Properties of reovirus RNA: (a) *Buoyant density:* The buoyant density of reovirus RNA as determined by equilibrium density gradient centrifugation in Cs_2SO_4 is *ca.* 1.61, in agreement with that reported for the double-stranded replicative forms of RNA-containing poliovirus[10] and bacteriophage MS2[11] (Fig. 1).

TABLE 1

EFFECT OF SALT CONCENTRATION ON THERMAL DENATURATION OF REOVIRUS RNA

SSC concentration	*Tm*, °C
1.0×	94
0.2×	87
0.1×	83
0.05×	81
0.02×	80
0.02× + 2.5 mM $MgCl_2$	>98
Deionized H_2O	98

RNA solutions at a concentration of 15–25 μg/ml in sealed cuvettes were heated slowly in a Gilford model 2000 spectrophotometer, and the changes in optical density at 260 mμ were recorded.

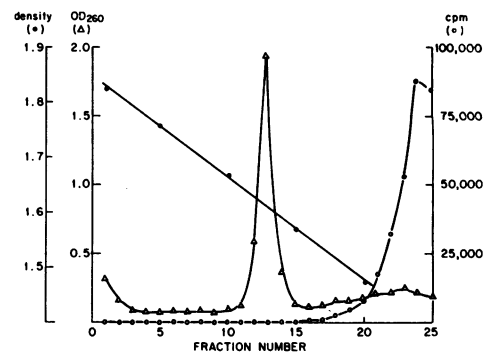

FIG. 1.—Failure of reovirus RNA to bind H^3-labeled actinomycin C_1.

(b) *Thermal denaturation:* Reovirus RNA undergoes a sharp thermal transition in the temperature range 80–100°C, with a 35 per cent increase in absorbancy at 260 mμ (Fig. 2). The T_m is lowered by decreasing the salt concentration, and the presence of Mg^{++} stabilizes the helical structure at elevated temperatures (Table 1). As reported by Gomatos and Tamm,[12] the T_m in deionized H_2O is 98°C; this may reflect a stabilizing effect of residual Mg^{++} in the absence of citrate. For example, RNA dissolved in 0.02 × SSC containing 2.5×10^{-3} *M* Mg^{++} showed no evidence of denaturation at 98°C in contrast to a T_m of 80° in the absence of added Mg^{++}.

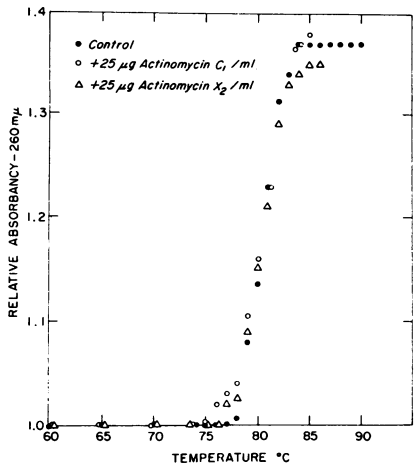

FIG. 2.—Thermal denaturation of reovirus RNA, 13 μg/ml.

(c) *Sedimentation coefficient:* Sedimentation analysis of the RNA revealed the presence of at least three components. The major component had a sedimentation coefficient of 12.1*S*, and additional peaks were observed at 14.2*S* and 10.7*S*. Similar values were obtained when a different RNA preparation which had not been purified by Cs_2SO_4 centrifugation was analyzed.

(d) *Sensitivity to enzymatic degradation:* Reovirus RNA labeled with uridine-H^3 was isolated from virus which had replicated in the presence of uridine-H^3 (0.16 μc/ml). When purified as described in *Experimental*, but prior to Cs_2SO_4 centrifugation, the RNA contained 720 cpm/μg. Reovirus RNA is resistant to digestion by DNase, exonucleases I and III of *E. coli*, spleen phosphodiesterase, and to ribonuclease in the presence of 1 × SSC or PBS. It is degraded to acid-soluble fragments by venom phosphodiesterase, micrococcal nuclease, and by RNase in the presence of 0.01–0.1 × SSC. After heating for 10 min at 100°C, the RNA is also degraded by spleen phosphodiesterase but not by DNase (Table 2).

Absence of interaction of reovirus RNA with actinomycin: When actinomycin complexes to helical DNA, there is an increase in the melting temperature,[13, 14] molecular weight,[15] and sedimentation coefficient,[16] and a decrease in the buoyant density[17] of the DNA. Furthermore, the antibiotic undergoes a shift in its absorption maximum and a reduction in its maximum absorbancy.[16, 18] Gomatos *et al.* have reported[2] that reovirus RNA did not shift the absorption maximum of actinomycin, although it lowered the absorbancy at the maximum by 2 per cent as compared to 25 per cent for a comparable quantity of salmon sperm DNA. As an additional test of the possible interaction of double-stranded RNA with actinomycin, its effects on the T_m and sedimentation coefficient of reovirus RNA were examined. Actinomycin C_1 or X_2 at a concentration of 25 μg/ml did not affect the T_m of 81°C measured in 0.05 × SSC (Fig. 2). An RNA solution which contained components of 12.1, 14.2, and 10.7*S* determined by sedimentation velocity centrifugation was mixed with actinomycin C_1, 20 μg/ml, and recentrifuged. Peaks were again observed at 12.4, 14.4, and 10.7*S*. A different RNA preparation not purified by Cs_2SO_4 centrifugation contained components with similar *S* values in the presence and absence of 20 μg/ml of actinomycin X_2.

A more direct test of the binding capacity of reovirus RNA was made with radioactive actinomycin. Reovirus RNA, 54 μg, and 11 μg actinomycin C_1-H^3 were incubated at room temperature in 0.2 ml SSC for 30 min, overlaid onto a Cs_2SO_4 solution of average density 1.60, and centrifuged for 5 days at 33,000 rpm in the Spinco SW-39 rotor. Fractions of 0.2 ml were collected through a hole punctured in the bottom of the tube. The distribution of RNA was determined from absorbancy measurements at 260 mμ. Actinomycin-H^3 was counted as described previously.[19] As shown in Figure 1, the buoyant density of the RNA, 1.61, was un-

TABLE 2

ENZYMIC DEGRADATION OF REOVIRUS RNA

Enzyme	Conditions	Acid-precipitable cpm	% Degraded
RNA only	1 × SSC	1880	—
RNase (0.4 μg/ml)	PBS	1700	10
"	2 × SSC	1690	10
"	1 × SSC	1640	13
"	0.1 × SSC	102	95
"	0.01 × SSC	305	84
RNA only	A	1580	—
Venom phosphodiesterase (10 μg/ml)	0.01 *M* tris, pH 8.6, + 30 mM $MgCl_2$	1640	0
Venom phosphodiesterase (50 μg/ml)	0.03 *M* tris, pH 8.8, + 30 mM $MgCl_2$	816	48
Spleen phosphodiesterase (0.5 units/ml)	0.1 × SSC	1550	2
DNase (5 μg/ml)	A + 10 mM $MgCl_2$	1600	0
DNase + venom phosphodiesterase (10 μg/ml)	A + 10 mM $MgCl_2$	1430	10
Exonuclease I (360 units/ml)	0.08 *M* glycine, pH 9.2, + 2.5 mM $MgCl_2$	1530	3
Exonuclease I (360 units/ml) + III (900 units/ml)	0.05 *M* tris, pH 8.6, + 2.5 mM $MgCl_2$	1590	0
RNase (2 μg/ml)	A	216	86
Micrococcal nuclease (7 μg/ml)	A + 0.5 mM $CaCl_2$	116	93
Micrococcal nuclease + spleen phosphodiesterase	A + 0.5 mM $CaCl_2$	301	81
Denatured RNA only	A	2080	—
DNase	A + 10 mM $MgCl_2$	2080	0
Venom phosphodiesterase (10 μg/ml)	0.01 *M* tris, pH 8.6, + 10 mM $MgCl_2$	1760	15
Venom phosphodiesterase (50 μg/ml)	0.01 *M* tris, pH 8.8, + 30 mM $MgCl_2$	730	65
Spleen phosphodiesterase	0.1 × SSC	184	91

Radioactive reovirus RNA (720 cpm/μg) was isolated from virus grown in cells incubated with uridine-H^3. RNA, 209 μg/ml in PBS, was diluted to a concentration of 20 μg/ml and incubated at 37° for 30 min. The 50 μg/ml venom phosphodiesterase digestion was incubated at 23°C with an RNA concentration of 0.2μg/ml. Acid-precipitable material was counted on filters as described for the polymerase standard assay. RNA was denatured by heating at 100°C for 10 min followed by quenching in an ice bath. Enzyme and $MgCl_2$ were then added to the appropriate vessels. "A" is 0.01 *M* tris buffer, pH 7.9, + 0.01 *M* NaCl. DNase was the electrophoretically purified enzyme.

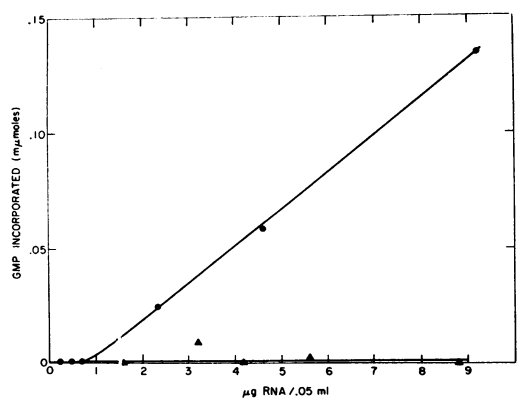

FIG. 3.—Template activity of reovirus RNA for *E. coli* RNA polymerase: (●) before, (▲) after purification by Cs_2SO_4 equilibrium density gradient centrifugation.

affected by the presence of actinomycin, and no binding of the radioactive antibiotic to reovirus RNA was observed. Actinomycin X_2 labeled with C^{14} was tested as above and failed to bind to reovirus RNA. The inability of double-stranded reovirus RNA to form complexes with actinomycin is not unexpected in view of the observation that helical RNA exists in the A configuration,[20] and actinomycin binds to DNA in the B form.[21]

RNA polymerase: Reovirus RNA prepared as described in *Experimental*, but not purified by Cs_2SO_4 density gradient centrifugation, was

tested for its template activity with *E. coli* RNA polymerase. The RNA stimulated the incorporation of nucleotides into an acid-insoluble product to an extent directly dependent upon the amount of RNA added (Fig. 3). Several RNA preparations were tested for template activity, and their activities were different. For example, template activity equivalent to 0.04 μg DNA in the standard assay required the addition of 39, 9.2, and 2.9 μg of three different RNA preparations. No stimulation was observed in the presence of less than 1 μg RNA, although concentrations of DNA in this range stimulate readily detectable levels of polynucleotide synthesis (see Tables 4 and 5). As reported previously,[2] actinomycin inhibited the template activity of DNA and unpurified RNA to the same extent, and both AMP and GMP incorporation were inhibited (Table 3).

After purification in a Cs_2SO_4 density gradient, reovirus RNA did not stimulate the incorporation of GMP (Fig. 3). No stimulation was observed when the enzyme concentration and incubation time were increased two- to threefold. Similarly, the incorporation of AMP (or CMP) was stimulated before, but not after, purifi-

TABLE 3

INHIBITION OF RNA POLYMERASE REACTION BY ACTINOMYCIN

Template	1 μg Actinomycin	AMP-H^3 ($m\mu$moles incorporated)	GMP-H^3	% Inhibition
2.7 μg L-929 DNA	−	1.40	...	..
	+	0.07	...	95
6.7 μg L-929 RNA	−	...	1.19	..
	+	...	0.21	82
2.3 μg Reovirus RNA	−	0.06	...	..
	+	0.01	...	83
2.9 μg Reovirus RNA	−	...	0.13	..
	+	...	0.03	77
3 μg Reovirus RNA (Cs_2SO_4-purified)	−	0.00	0.00	..

Conditions of incubation and analysis were as described in *Experimental*. The specific activities of ATP-H^3 and GTP-H^3 were 9.7 and 25 μc/μmole, respectively.

TABLE 4

EFFECT OF PURIFIED REOVIRUS RNA ON RNA POLYMERASE REACTION

Additions	$m\mu$Moles GMP-H^3 incorporated
22 μg Reovirus RNA	0.00
1 μg Calf thymus DNA	0.41
1 μg Calf thymus DNA + 5.5 μg reovirus RNA	0.33
1 μg Denatured thymus DNA	0.24
1 μg Denatured thymus DNA + 5.5 μg reovirus RNA	0.22
0.9 μg dGdC	0.24
0.9 μg dGdC + 5.5 μg reovirus RNA	0.28

The standard assay mixture in a volume of 0.3 ml was supplemented with 7.5 μmoles sodium phosphoenolpyruvate (PEP) and 5 μg PEP kinase. After, incubation for 30 min at 37°, the vessels were chilled. Sodium nucleate and bovine serum albumin, 2 mg each, 4 μmoles GTP, and 5 ml 5% TCA were then added. After 15 min the vessels were centrifuged, the pellet was washed three times with 5% TCA, and dissolved in 0.5 ml formic acid. Four ml of ethanol was added followed by 15 ml liquifluor, and the solutions were counted in a scintillation counter. Thymus DNA was denatured by heating at 100°C for 10 min in 0.01 *M* tris buffer, pH 7.9, 0.01 *M* NaCl followed by quenching in ice. This experiment was performed with a preparation of RNA polymerase, fraction IV, obtained from A. Cerami and D. Ward.

TABLE 5

ADDITIVE TEMPLATE ACTIVITY OF DNA AND RNA FOR RNA POLYMERASE

Additions	$m\mu$Moles GMP-H^3 incorporated
0.034 μg L-929 DNA	0.07
6.87 μg Reovirus RNA	0.11
0.034 μg L-929 DNA + 6.87 μg reovirus RNA	0.17

The reovirus RNA was not Cs_2SO_4-banded. Standard assay conditions. GTP-H^3 specific activity = 25 μc/μmole.

cation of reovirus RNA in Cs_2SO_4 gradients (Table 3). Exposure of unbanded reovirus RNA preparations to Cs_2SO_4 did not *per se* destroy their template activity; no change in activity was detected after incubation at 4°C for 40 hr in Cs_2SO_4 (ρ = 1.60) followed by extensive dialysis against 0.01 *M* tris buffer pH 7.9. Furthermore, Cs_2SO_4-banded RNA at a concentration five times that of native or denatured calf thymus DNA or of dGdC did not affect their template activities, indicating that the purified RNA does not contain an inhibitor of the polymerase reaction (Table 4). On the other hand, RNA not previously purified in Cs_2SO_4 together with DNA at low concentrations promoted incorporation to an extent equal to the sum of the individual nucleic acids (Table 5).

The above results suggest that reovirus RNA does not serve as a template for RNA polymerase. Instead, it seems likely that the RNA preparations contain small and variable amounts of DNA which band at a density of *ca.* 1.4 in Cs_2SO_4[22] and are thus removed by equilibrium centrifugation. The following additional control experiments were performed to test this conclusion. (1) Reovirus RNA was purified as described in *Experimental* from HeLa S3-1 cells which had been labeled before virus infection by growth for 48 hr in media containing 0.16 μc/ml of thymidine-H^3 (methyl-labeled, specific activity = 13 c/mmole). Before Cs_2SO_4 centrifugation the RNA solution of 595 μg/ml contained 270 cpm/ml; 5.9 μg of this RNA stimulated the incorporation of 0.04 mμmole of CMP-H^3 in the RNA polymerase standard assay. After purification in Cs_2SO_4 no radioactivity could be detected in a solution containing 255 μg RNA/ml; 3.3 μg RNA did not promote the incorporation of CMP. (2) Neither DNase I nor exonuclease I degrades reovirus RNA (Table 2). Pretreatment and 9.2 μg reovirus RNA (not Cs_2SO_4-purified) with 5 μg/ml electrophoretically purified DNase (30 min, 37°C) reduced its template of activity 0.05 in the standard assay from 0.17 to 0.11 mμmole GMP incorporated. Similarly, exposure to *ca.* 360 units/ml of exonuclease I (DEAE-cellulose fraction) for 30 min at 37°C in 0.05 *M* glycine buffer, pH 9.2, 2.5×10^{-3} *M* $MgCl_2$, reduced the stimulation of GMP incorporation by 70 per cent. (3) Radioactive reovirus RNA-H^3, 478 μg/ml and 43 cpm/μg, was incubated in 0.3 *N* NaOH for 16 hr at 37°C. The digestion of RNA was complete as judged by the loss of all acid-precipitable radioactivity. The hydrolysate was then dialyzed exhaustively against 0.01 *M* tris buffer, pH 7.9. The dialyzed digest at a $^1/_{20}$ dilution had no measurable absorbancy at 260 mμ. A volume equivalent before digestion to 9.2 μg RNA was assayed with RNA polymerase, and found to direct the incorporation of 0.06 mμmoles as compared with 0.11 mμmole CMP prior to alkaline hydrolysis. Treatment with 4 per cent TCA at 90°C for 15 min abolished all template activity. (4) If the template activity of reovirus RNA preparations were due to contaminating DNA, then more exhaustive purification of the virus prior to extraction of viral RNA might directly yield RNA devoid of template activity even before ethanol precipitation and Cs_2SO_4 centrifugation. This has proved to be so.[23]

DNA polymerase: The results of an examination of the priming ability of reovirus RNA for DNA polymerase are shown in Table 6. As observed for RNA polymerase, reovirus RNA has no priming activity after purification in Cs_2SO_4.

Discussion and Conclusions.—The properties of reovirus RNA isolated by equilibrium density gradient centrifugation closely resemble those previously reported for reovirus RNA not purified in Cs_2SO_4. Both preparations contain two or more

TABLE 6

EFFECT OF PURIFIED REOVIRUS RNA ON DNA POLYMERASE

Additions	mμMoles dTMP-H³ incorporated
14 μg Reovirus RNA	0.00
15 μg Calf thymus DNA	0.76
14 μg Reovirus RNA + 15 μg thymus DNA	0.72

components with sedimentation coefficients at 11–14*S*.[24] The RNA's are relatively resistant to RNase digestion, and undergo a sharp thermal denaturation in the range 80–100°C.[12] Furthermore, reovirus RNA has a buoyant density of 1.61 in Cs_2SO_4, similar to other double-stranded RNA's.[10, 11]

Although centrifugation in Cs_2SO_4 does not detectably affect the physical properties of reovirus RNA, this procedure abolished any activity for polynucleotide synthesis associated with this RNA. The experiments described above indicate that the loss of activity does not result from exposure of the RNA to Cs_2SO_4 as such, and is not due to the presence of an inhibitor of the polymerases. Instead, it appears more likely that the activity before Cs_2SO_4 purification can be ascribed to the presence of some host mouse cell DNA in the RNA preparations. This is in accord with the failure of MS2 replicative form to serve as a template for RNA polymerase.[25]

It is of interest that the individual nearest-neighbor determinations of RNA polymerase product in a "reovirus RNA-primed synthesis"[26] are, within the limits of precision of the method, indistinguishable from those reported for mouse DNA,[27] and that the ratio (A + U)/(G + C) derived from these data yields a value of 1.43. This value is identical with that obtained from mouse DNA itself,[27] and differs from the chemically determined ratio, 1.27, reported for reovirus RNA.[28]

If reovirus RNA does not serve as a template for the normal cell polymerase, then it is reasonable to expect that a new virus-specific replication system is produced in the course of infection. The inhibitory effect of actinomycin on reovirus multiplication suggests that the antibiotic is affecting cellular activities required for virus development.

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[23] For this experiment the virus was prepared as described in *Experimental*, but subjected to an additional cycle of equilibrium centrifugation in CsCl; it was then incubated with micrococcal nuclease (20 μg/ml; Tris buffer 0.05 *M*, pH 8.6; 0.01 *M* Ca^{++}) for 7 hr at room temperature, followed by overnight dialysis against Tris buffer 0.01 *M* pH 7.9, brought to 0.15 *M* NaCl–0.01 *M* phosphate buffer pH 7.4, and incubated with crystalline pancreatic DNase (200 μg/ml; 0.01 *M* Mg^{++}; $1^1/_2$ hr at room temperature and 19 hr at 4°C). After this treatment the virus was again dialyzed against PBS, harvested by centrifugation, and the RNA extracted with phenol. The T_m of this RNA was identical with that obtained in the usual way, and it contained components of 10.6, 12.5, and 14.9*S*. With RNA polymerase in a standard assay 3.5 μg of this RNA directed the incorporation of 0.005 mμmole of radioactive CMP into acid-precipitable material; under the same conditions 4 μg L-cell DNA promoted synthesis of 0.74 mμmole CMP into polynucleotide.
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*NONCONSERVATIVE DNA REPLICATION IN BACTERIA AFTER THYMINE STARVATION**

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The phenomenon of thymineless death, first described over ten years ago by Barner and Cohen,[1] remains poorly understood, although much information about it has been accumulated. The loss in viability during thymine deprivation was initially ascribed to unbalanced growth,[1] as cytoplasmic syntheses continue in the absence of chromosomal replication. This concept was subsequently refined when it was shown that specifically RNA synthesis, but not protein synthesis, is a prerequisite for thymineless death.[2, 3] Cells that have completed a round of DNA replication but have not initiated the next round are immune to the pathological effects of thymine deprivation;[4] thus, a direct involvement with the chromosomal replication cycle is apparent.

In addition to lethality, many other events have been correlated with thymine deficiency. Thymine deprivation is mutagenic[5–8] and recombinogenic.[9–11] It induces the production of colicins,[12] effects the induction (or diversion) of prophage,[13, 14] and causes the premature initiation of the DNA-replication cycle.[15] Many of these consequences of thymine deprivation parallel those of ultraviolet (UV) irradiation; and, in fact, a synergism has been observed between thymineless