Reprinted from the PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES
Vol. 54, No. 3, pp. 919–927. September, 1965.

*THE SYNTHESIS OF A SELF-PROPAGATING AND INFECTIOUS NUCLEIC ACID WITH A PURIFIED ENZYME**

BY S. SPIEGELMAN, I. HARUNA, I. B. HOLLAND, G. BEAUDREAU, AND D. MILLS†

DEPARTMENT OF MICROBIOLOGY, UNIVERSITY OF ILLINOIS, URBANA

Communicated July 23, 1965

The unambiguous analysis of a replicating mechanism demands evidence that the reaction being studied is, in fact, generating replicas. If, in particular, the concern is with the synthesis of a viral nucleic acid, data on base composition and nearest neighbors are not sufficient. Ultimately, proof must be offered that the polynucleotide product contains the information necessary for the production of the corresponding virus particle in a suitable test system.

These conditions impose severe restraints on the type of experiments acceptable as providing information which is irrefutably relevant to the nature of the replicating mechanism. Clearly, the enzyme system employed must be free of interfering and confounding activities so that the reaction can be studied in a simple mixture

containing only the required ions, substrates, and templates. Since the biological activity of the product is likely to be completely destroyed by even one break, the elimination of nuclease activity must be rigorous indeed. The purity required imposes the necessity that the enzymological aspects of the investigation be virtually completed before an examination of mechanism can be safely instituted.

We have previously reported[1] the purification of two distinct RNA-dependent-RNA-polymerases (designated "replicases" for brevity) induced in the same host by two unrelated[2, 3] RNA bacteriophages (MS-2 and Qβ). It was shown that under optimal conditions, both enzymes are virtually inactive with a variety of heterologous RNA species, including ribosomal and sRNA of the host. Further, neither replicase can function with the other's RNA. Each enzyme recognizes the RNA genome of its origin and requires it as a template for normal synthetic activity.

In summary, the purified replicases exhibited[1] the following distinctive features: (*a*) freedom from detectable levels of the DNA-dependent-RNA-polymerase, ribonuclease I,[4] ribonuclease II,[5] and RNA phosphorylase; (*b*) complete dependence on added RNA for synthetic activity; (*c*) competence for prolonged (more than 5 hr) synthesis of RNA; (*d*) ability to synthesize many times the input templates; (*e*) saturation at low levels of RNA (1 γ RNA/40 γ protein); (*f*) virtually exclusive requirement for intact homologous template under optimal ionic conditions.

The discriminating selectivity of the replicase permitted a simple test of similarity between template and product. Haruna and Spiegelman[6] showed that when reactions are started at template concentrations below those required to saturate the enzyme, RNA synthesis follows an autocatalytic curve. When the saturation concentration level is reached, the kinetics become linear. The autocatalytic behavior below saturation of the enzyme implies that the newly synthesized product can in turn serve as templates for the reaction. To test this conclusion directly, the product was purified from a reaction allowed to proceed until a 65-fold increase of the input RNA had accumulated. The ability of the newly synthesized RNA to initiate the reaction was examined in a saturation experiment and found to be identical to RNA isolated from virus particles. It is evident that the sequences employed by the enzyme for recognition are being faithfully copied.

The findings summarized above and the state of purity of the enzymes encouraged us to enter the next phase of the investigation and examine the infectivity of the synthesized material. It is the purpose of the present paper to describe experiments demonstrating that the RNA produced by replicase is fully competent to program the production of complete virus particles. The data establish that the reaction being studied is indeed generating self-propagating replicas of the input RNA.

Materials and Methods.—(1) *Biological system and enzyme preparation:* The bacterial virus employed is Qβ, isolated by Watanabe.[2] The host and assay organism is a mutant Hfr strain of *E. coli* (Q13) isolated in the laboratory of W. Gilbert by Diane Vargo. This bacterial strain has the convenient property[7] of lacking ribonuclease I and RNA phosphorylase. The preparation of infected cells and the subsequent isolation and purification of the replicase follows the detailed protocol of Haruna and Spiegelman.[1] The preparation of virus stocks and the purification of RNA from them follow the methods of Doi and Spiegelman.[8]

(2) *The assay of enzyme activity by incorporation of radioactive nucleotides:* The standard reaction mixture is 0.25 ml and, in addition to 40 γ of enzyme, contains the following in μmoles: Tris HCl, pH 7.4, 21; $MgCl_2$, 3.2; CTP, ATP, UTP, and GTP, 0.2 each. The reaction is ter-

minated in an ice bath by the addition of 0.15 ml of neutralized saturated pyrophosphate, 0.15 ml of neutralized saturated orthophosphate, and 0.1 ml of 80% trichloracetic acid. The precipitate is transferred to a membrane filter and washed 7 times with 5 ml of cold 10% TCA. The membrane is then dried and counted in a liquid scintillation counter as described previously. UTP^{32} was synthesized as described by Haruna *et al.*[9] It was used at a specific activity such that the incorporation of 20,000 cpm corresponds to the synthesis of 1 γ of RNA, permitting the use of 20 λ samples for following the formation of labeled RNA.

(3) *Isolation of synthesized product:* Samples removed from the reaction mixture are placed immediately in an ice bath and 20 λ removed for immediate assay of radioactive RNA as described in (2) above. The volume is then adjusted to 1 ml with TM buffer (10^{-2} *M* Tris, 5×10^{-3} *M* $MgCl_2$, pH 7.5). One ml of water-saturated phenol is then added and the mixture shaken in heavy wall glass centrifuge tubes (Sorvall, 18 × 102 mm) at 5°C for 1 hr. After separation of the water phase from the phenol by centrifugation at 11,000 rpm for 10 min, another 1 ml of TM buffer is added to the phenol which is then mixed by shaking for 15 min at 5°C. Again, the phenol and water layers are separated, and the two water layers combined. Phenol is eliminated by two ether extractions, care being taken to remove the phenol from the walls of the centrifuge tubes by completely filling them with ether after each extraction. The ether dissolved in the water phase is then removed with a stream of nitrogen. The RNA is precipitated by adding $^1/_{10}$ vol of potassium acetate (2 *M*) and 2 vol of cold absolute ethanol. The samples are kept for 2 hr at −20°C before being centrifuged for one hour at 14,000 rpm in a Sorvall SS 34 rotor. The pellets are drained, and the remaining alcohol is removed by storing under reduced pressure in a vacuum desiccator for 6–8 hr at 5°C. The RNA is then dissolved in 1 ml of buffer (10^{-2} *M* Tris, 10^{-2} *M* $MgCl_2$, pH 7.5) and samples are removed immediately for infectivity assay. TCA-precipitable radioactivity is measured on 20 λ aliquots of the final product from which the per cent recovery of synthesized RNA can be determined. In the range of 1–8 γ, it was found that, in general, 65% of the synthesized RNA was recovered. All purified products were examined for the presence of intact virus particles by assay on whole cells and none were found.

(4) *The assay for infectivity of the synthesized RNA:* The procedure used is a modification of the spheroplast method of Guthrie and Sinsheimer.[10] The necessary components are as follows:

(a) *Medium:* The medium used is a modification of the 3XD medium of Fraser and Jerrel[11] and requires in grams/liter the following: Na_2HPO_4, 2 gm; KH_2PO_4, 0.9 gm; NH_4Cl, 1 gm; glycerol (Fisher reagent), 30 gm; Difco yeast extract, 50 mg; casamino acids (Difco vitamin-free), 15 gm; L-methionine, 10 mg; D,L-leucine, 10 mg; $MgSO_4 \cdot 7\ H_2O$, 0.3 gm. These components are mixed in the order indicated in 500 ml glass-distilled water. To this is finally added another 500 ml containing 0.3 ml *M* $CaCl_2$.

(b) *Sucrose nutrient broth* (*SNB*) contains in grams/liter the following: casamino acids (Difco), 10 gm; nutrient broth (Difco), 10 gm; glucose, 1 gm; sucrose, 100 gm. After autoclaving, the following are added aseptically: 10 ml 10% $MgSO_4$, and 3.3 ml 30% bovine serum albumin (BSA) from Armour Laboratories.

(c) *Reagents required for the production of spheroplasts:* The followings olutions are required for the production of spheroplasts: lysozyme (Sigma) at 2 mg/ml in 0.25 *M* Tris, pH 8.0; protamine sulfate from Eli Lilly and Co., 0.1%; and sterile solutions of 30% BSA; 0.25 *M* Tris (Trizma), pH 8.0; 0.01 *M* Tris, pH 7.5 and pH 8.0; 0.5 *M* sucrose; 0.4% EDTA in 0.01 *M* Tris, pH 7.5.

For the preparation of spheroplasts, an overnight culture of Q13 in 3XD medium is first diluted into a fresh medium to an OD_{660} of 0.06. The culture is allowed to grow to an OD_{660} between 0.2 and 0.22 at 30°C, and the cells are spun down at room temperature. The pellet from 25 ml of cells is first suspended in 0.35 ml of 0.5 *M* sucrose plus 0.1 ml of 0.25 *M* Tris, pH 8.0. Then 0.01 ml of lysozyme is added followed by 0.03 ml EDTA. After 10 min at room temperature, when conversion to spheroplasts is 99.9%, 0.2 ml of this stock is diluted into 3.8 ml SNB, and 0.025 ml of protamine sulfate is added. The spheroplast stock must be examined microscopically before proceeding. The presence of even 5% breakage of spheroplasts indicates a preparation which will give a low efficiency of plating. In agreement with Paranchych,[12] we have found that protamine increases the efficiency of detection of infectious RNA. However, the optimal protamine concentration in the present system is considerably lower than that used by Paranchych.

The RNA infection is usually carried out at room temperature with solutions containing 0.5 γ of RNA/ml, a concentration at which the assay is not limited by the number of spheroplasts per

infectious unit. To 0.2 ml of RNA is added 0.2 ml of the spheroplast stock containing about 3×10^7 spheroplasts. The samples are mixed, and immediately an aliquot is removed and diluted appropriately through SNB before plating on L-agar using Q13 as the indicator. The soft agar (0.7%) layer employed (2.5 ml) contains 10% sucrose; 0.1% $MgSO_4$; and 0.01 ml of 30% BSA per tube plus 0.2 ml of an overnight culture of Q13. To obtain reproducibility, the spheroplast stock is used 15–45 min after dilution into the SNB. Efficiency of plating (e.o.p.) is usually $2\text{–}8 \times 10^{-7}$. Higher efficiencies ($>1 \times 10^{-6}$) can be obtained if the spheroplast stock is employed immediately after dilution and by including a stabilization period in the SNB dilution tubes, rather than plating immediately. However, this higher plating efficiency decays rapidly, making it difficult to obtain reproducible duplicates in repetitive assays. Since reproducibility was of greater concern than efficiency, the assay method detailed above was employed.

Results.—In designing experiments which involve infectivity assays of the enzymatically synthesized RNA, it is important to recognize that even highly purified enzymes from infected cells, although demonstrably devoid of intact cells, are likely to include some virus particles. Chemically, the contamination is trivial, amounting to 0.16 γ of nucleic acid and 0.8 γ of protein for each 1,000 γ of enzyme protein employed in the present studies. Since 40 γ of protein are used for each 0.25 ml of reaction, the contribution to the total RNA by the particles is only 0.006 γ, which is to be compared with the 0.2 γ of input RNA and the 3–20 γ synthesized in the usual experiment. It was shown in control experiments that RNA freshly extracted from particles in the reaction mixture is no more infective than that obtained from the usual purified virus preparation. Further, the mandatory requirement for added RNA proves that, within the incubation times used, this small amount of RNA is either inadequate or unavailable for the initiation of the reaction. Thus, these particles do not significantly influence either the chemical or the enzymatic aspects of the experiment. However, because of their higher infective efficiency, even moderate amounts of intact virus cannot be tolerated in the examinations of the synthesized RNA for infectivity. Consequently, all RNA preparations were phenol-treated [*Methods*, (3)] prior to assay. Further, the phenol-purified RNA was routinely tested for whole virus particles and none were found in the experiments reported.

We now undertake to describe experiments in which the kinetics of the appearance of new RNA and infective units were examined in two different ways. The first shows that the accumulation of radioactive RNA is accompanied by a proportionate increase in infective units. The second type proves by a serial dilution experiment that the newly synthesized RNA is infective.

(1) *Assay of infectivity of the purified product:* To compare the appearance of new RNA and infectious units in an extensive synthesis, 8 ml of reaction mixture was set up containing the necessary components in the concentrations specified in *Methods* (2). Aliquots were taken at the times indicated for the determination of radioactive RNA and purification of the product for infectivity assay. The results are summarized in Figure 1 in the form of a semilogarithmic plot against time of the observed increase in both RNA and infectious units. Further details of the experimental protocol are given in the corresponding legend.

The amount of RNA (0.8 γ/ml) put in at zero time is well below the saturation level of the enzyme present.[6] Consequently, the RNA increases autocatalytically for about the first 90 min, followed by a synthesis which is linear with time, a feature which had been observed previously.[6] It will be noted that the increase in RNA is

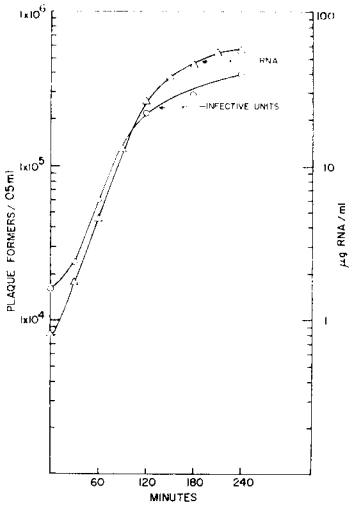

FIG. 1.—Kinetics of RNA synthesis and formation of infectious units. An 8-ml reaction mixture was set up containing the components at the concentrations specified in *Methods* (2). Samples were taken as follows: 1 ml at 0 time and 30 min, 0.5 ml at 60 min, 0.3 ml at 90 min, and 0.2 ml at all subsequent times. 20 λ were removed for assay of incorporated radioactivity as described in *Methods* (2). The RNA was purified from the remainder [*Methods* (3)], radioactivity being determined on the final product to monitor recovery. Infectivity assays were carried out as in *Methods* (4).

paralleled by a rise in the number of infectious units. During the 240 min of incubation, the RNA experiences a 75-fold increase, and the infectious units experience a 35-fold increase over the amount present at zero time. These numbers are in agreement within the accuracy limits of the infectivity test. Experiments carried out with other enzyme preparations yielded results in complete accord with those just described.

It is clear that one can provide evidence for an increase in the number of infectious units which parallels the appearance of newly synthesized RNA.

(2) *Proof that the newly synthesized RNA molecules are infective:* The kind of experiments just described offer plausible evidence for infectivity of the radioactive RNA. They are not, however, conclusive, since they do not eliminate the possibility that the agreement observed is fortuitous. One could argue that the enzyme is "activating" the infectivity of the input RNA while synthesizing new noninfectious RNA and that the rather complex exponential and linear kinetics of the two processes happen to coincide by chance.

Direct proof that the newly synthesized RNA is infectious can in principle be obtained by experiments which use N^{15}-H^3-labeled initial templates to generate N^{14}-P^{32}-labeled product. The two can then be separated[8] in equilibrium density gradients of Cs_2SO_4. Such experiments have been carried out for other purposes, and will be described elsewhere. However, the steepness of the Cs_2SO_4 density gradients makes it difficult to achieve a separation clean enough to be completely satisfying.

There exists, however, another approach which bypasses these technical difficulties and takes advantage of the fact that we are dealing with a self-propagating entity. Consider a series of tubes, each containing 0.25 ml of the standard reaction mixture, but no added template. The first tube is seeded with 0.2 γ of Qβ-RNA and incubated for a period adequate for the synthesis of several γ of radioactive RNA. An aliquot (50 λ) is then transferred to the second tube which is in in turn permitted to synthesize about the same amount of RNA, a portion of which is again transferred to a third tube, and so on. If each successive synthesis pro-

TABLE 1

SERIAL TRANSFER EXPERIMENT

1 Transfer no.	2 Interval (min)	3 Time	Formation of RNA: 4 Cpm × 10^{-3}	5 Total (γ)	6 Δ (γ)	7 Σ (γ)	Concentration of Original Template: 8 γ	9 Strands	10 IU	Formation of IU: 11 Δ × 10^{-5}	12 Σ × 10^{-5}	13 Observed e.o.p × 10^{-7}	14 % Recovery of P^{32}-RNA
0	0	0	0	0.2	0	0	2.0×10^{-1}	1.2×10^{11}	6.0×10^{4}	1.0	1.0	5.5	...
1	40	40	64	3.2	3.0	3.0	2.0×10^{-1}	1.2×10^{11}	6.0×10^{1}	5.2	5.2	3.2	54.2
2	40	80	84	4.2	3.6	6.6	4.0×10^{-2}	2.4×10^{10}	1.2×10^{1}	2.2	6.5	2.0	88.3
3	40	120	112	5.7	4.9	11.5	6.7×10^{-3}	4.0×10^{9}	2.0×10^{0}	11.3	17.4	5.3	59.9
4	40	160	134	6.7	5.6	17.1	1.1×10^{-3}	6.6×10^{8}	3.3×10^{-1}	5.7	21.2	3.0	42.3
5	30	190	113	5.7	4.4	21.5	1.9×10^{-4}	1.1×10^{8}	55	7.4	27.6	3.0	63.4
6	30	220	144	7.2	6.1	27.6	3.1×10^{-5}	1.8×10^{7}	9	15.0	36.4	3.7	82.4
7	30	250	150	7.5	6.1	33.7	5.1×10^{-6}	3.0×10^{6}	1.5	13.4	48.1	5.0	52.9
8	30	280	162	8.1	6.6	40.3	8.6×10^{-7}	5.0×10^{5}	<1	8.8	54.7	5.2	51.4
9	30	310	164	8.2	6.6	46.9	1.4×10^{-7}	8.4×10^{3}	<1	5.6	58.4	2.0	92.6
10	30	340	156	7.8	6.2	53.1	2.4×10^{-8}	1.4×10^{3}	<1	9.3	66.8	4.0	54.2
11	20	360	134	6.7	5.1	58.2	4.0×10^{-9}	2.3×10^{2}	<1	6.3	73.7	3.7	74.3
12	20	380	121	6.0	4.7	62.9	6.6×10^{-10}	3.8×10^{1}	<1	6.9	84.3	7.0	46.8
13	20	400	123	6.1	4.9	67.8	1.1×10^{-10}	6	<1	3.6	89.4	4.0	49.2
14	20	420	118	5.9	4.7	72.5	1.8×10^{-11}	1	<1	10.8	102.0	5.7	79.7
15	20	440	75	3.6	2.4	74.9	3.1×10^{-12}	0.16	<1	3.2	105.0	5.5	65.4

Sixteen reaction mixtures of 0.25 ml were set up, each containing 40 γ of protein and the other components specified for the "standard" assay in *Methods*. 0.2 γ of template RNA were added to tubes 0 and 1; RNA was extracted from the former immediately, and the latter was allowed to incubate for 40 min. Then 50 λ of tube 1 were transferred to tube 2, which was incubated for 40 min, and 50 λ of tube 2 then transferred to tube 3, and so on, each step after the first involving a 1–6 dilution of the input material. Every tube was transferred from an ice bath to the 35°C water bath a few minutes before use to permit temperature equilibration. After the transfer from a given tube, 20 λ were removed to determine the amount of P^{32}-RNA synthesized, and the product was purified from the remainder as described in *Methods*. Control tubes incubated for 60 min without the addition of the 0.2 γ of RNA showed no detectable RNA synthesis, nor any formation of infectious units.

All recorded numbers are normalized to 0.25 ml. Columns 1, 2, and 3 give the transfer number, the time interval permitted for synthesis, and the elapsed time from zero, respectively. Column 4 records the amount of radioactive RNA found in each tube at the end of the incubation, column 5 the total RNA in each, and 6 gives the net synthesis during the time interval. Column 7 lists the cumulative synthesis of RNA. The decreasing concentrations of the input RNA resulting from the serial dilutions are recorded in terms of γ (col. 8), number of strands (col. 9), and infectious units (IU) per tube (col. 10). The last is calculated from column 9 and from an efficiency of plating (e.o.p.) of 5×10^{-7}. Column 11 lists the increment in infectious units observed during each period of synthesis, corrected for the efficiency of recovery (col. 14), and column 12 represents the corresponding sum. Column 13 is the plating efficiency (e.o.p.) determined from the observed number of plaques (col. 11) and the actual amount of RNA assayed as determined from columns 6 and 14. Column 14 is determined from assays of acid-precipitable radioactivity on 20 λ aliquots of the final product as compared with column 5.

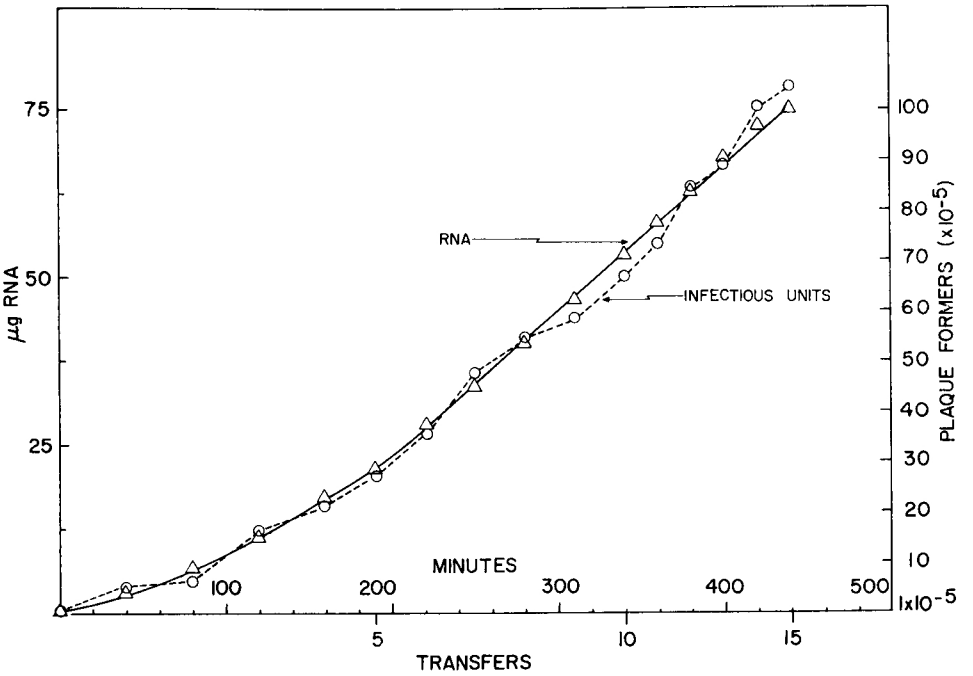

FIG. 2.—RNA synthesis and formation of infectious units in a serial transfer experiment. All details are as described in the heading to Table 1, and the data are taken from columns 7 and 11 and plotted against elapsed time (col. 3) and corresponding transfer number (col. 1). Both ordinates refer to amounts found in 0.25-ml aliquots.

duces RNA which can serve to initiate the next one, the experiment can be continued until a point is reached at which the initial RNA of tube 1 has been diluted to an insignificant level. In fact, enough transfers can be made to ensure that the last tube contains less than one strand of the input primer. *If in all the tubes, including the last, the number of infectious units corresponds to the amount of radioactive RNA found, convincing evidence is offered that the newly synthesized RNA is infectious.*

Table 1 records a complete account of such a serial transfer experiment and the corresponding legend provides the details necessary to follow the assays and calculations. Sixteen tubes are involved, the first (tube 0) bein gan unincubated zero time control. It will be noted that the successive dilution was such (1–6) that, by the 8th tube, there was less than one infectious unit ascribable to the initiating 0.2 γ of RNA. Nevertheless, this same tube showed 8.8×10^5 newly synthesized infectious units during the 30 min of its incubation. Finally, tube 15, which contained less than one strand of the original input, produced 1.4×10^{12} new strands and 3.2×10^5 infectious units in 20 min. It should be noted that a control tube lacking added RNA was incubated for 60 min. As compared with tube 1, which incorporated 4800 cpm for each 20 λ in 40 min, the control showed no increase above the zero time level of 80 cpm. Further, no synthesis of infective units was observed in such controls.

Figure 2 compares the cumulative increments with time in newly synthesized RNA (column 7) and infectious units (column 12). The agreement between increments in synthesized RNA and newly appearing infectious units is excellent at

TABLE 2

SEROLOGICAL BEHAVIOR OF VIRUS FORMED IN RESPONSE TO "SYNTHETIC" RNA

	Antisera					
	Anti-Qβ			Anti-MS-2		
Virus	0 Time	10 Min	% Survivors	0 Time	10 Min	% Survivors
Authentic Qβ	1.9×10^8	1.0×10^5	0.052	1.1×10^8	1.06×10^8	96
Virus from synthetic RNA	1.5×10^8	8.8×10^4	0.053	1.5×10^8	1.40×10^8	93

In all cases, lysates were made from *E. coli* Q13, which was also the assay organism. Antisera were used at 1/100 dilution, and the incubation temperature was 35°C. The numbers represent plaque formers per ml.

every stage of the serial transfer—and continues to the last tube. Long after the initial RNA has been diluted to insignificant levels, the RNA from one tube serves to initiate synthesis in the next. Further, as may be seen from the comparative constancy of the infective efficiency (Fig. 2 and column 13 of Table 1), the new RNA is fully as competent as the original viral RNA to program the snythesis of viral particles in spheroplasts.

To complete the proof, it was necessary to show that the viruses produced by the synthesized RNA were indeed Qβ, the original source of the RNA used as a seed in tube 1 to initiate the transfer experiment. Since Qβ is a unique serological type,[2, 3] this characteristic was chosen as a convenient diagnostic test. Plaques induced by the RNA synthesized in tube 15 were used to produce lysates, and the resulting particles exposed to antisera against MS-2 and Qβ. The results, briefly summarized in Table 2, show clearly that the synthetic RNA induces virus particles of the same serological type as authentic Qβ.

Discussion.—One perhaps might have imagined that an enzyme carrying out a complex copying process would show a high error frequency when functioning in the unfamiliar environment provided by the enzymologist. Had this been a quantitatively significant complication, biologically inactive strands should have accumulated as the synthesis progressed. That this is not the case is rather dramatically illustrated by the serial transfer experiment (Table 1 and Fig. 2). The RNA synthesized after the 15th transfer is as competent biologically as the initiating "natural" material derived from virus particles.

The successful synthesis of a biologically active nucleic acid with a purified enzyme is itself of obvious interest. However, the implication which is most pregnant with potential usefulness stems from the demonstration that the replicase is, in fact, generating identical copies of the viral RNA. For the first time, a system has been made available which permits the unambiguous analysis of the molecular basis underlying the replication of a self-propagating nucleic acid. Every step and component necessary to complete the replication must be represented in the reaction mixture described. If two enzymes are required,[13] both must be present and it should be possible either to establish their existence or to prove that one is sufficient. If an intermediate "replicating" stage intervenes between the template and the ultimate identical copy,[13] then a "replicative form" should be demonstrably present in the reaction mixture. If copying is direct, no such intermediate will be found. These and other issues of the replicating mechanism will be discussed in a subsequent publication which will detail the relevant experiments.

Summary.—Experiments are described with a purified RNA-dependent-RNA-polymerase (replicase) induced in *E. coli* by the RNA bacteriophage Qβ.

The data demonstrate that the enzyme can generate identical copies of added viral RNA. A serial dilution experiment established that the newly synthesized RNA is fully as competent as the original viral RNA to program the synthesis of viral particles and to serve as templates for the generation of more copies. Since the data show that the enzyme is, in fact, generating replicas, an unambiguous analysis of the RNA replicating mechanism is now possible in a simple system consisting of purified replicase, template RNA, ribosidetriphosphates, and Mg^{++}.

* This investigation was supported by USPHS research grant CA-01094 from the National Cancer Institute and grant GB-2169 from the National Science Foundation.

† Predoctoral trainee in Microbial and Molecular Genetics, grant USPHS 2-T1-GM-319.

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