

**REPLICATION OF BACTERIOPHAGE RIBONUCLEIC ACID:
ALTERATIONS IN POLYRIBOSOME PATTERNS AND ASSOCIATION
OF DOUBLE-STRANDED RNA WITH POLYRIBOSOMES IN
ESCHERICHIA COLI INFECTED WITH BACTERIOPHAGE R17***

BY BARBARA HOTHAM-IGLEWSKI† AND RICHARD M. FRANKLIN

PUBLIC HEALTH RESEARCH INSTITUTE OF THE CITY OF NEW YORK, INC.

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Double-stranded RNA isolated from cells infected with the RNA bacteriophage R17 has been implicated in the replicative mechanism of viral RNA.¹ Two types of double-stranded RNA have been described in cells infected with RNA viruses: the intact double strand of molecular weight 2×10^6 ,^{2, 3} known as *replicative form*,⁴ and the population of double-stranded molecules containing nascent single strands, known as *replicative intermediate*.⁵ The description of replicative intermediate isolated from infected cells by phenol extraction and its relation to *in vitro* replicating RNA structures has been discussed.¹⁻³ Besides removal of the RNA-dependent RNA polymerase from the replicating structure, phenol could possibly dissociate some of the hydrogen-bonded single strands from the double strand.³ Therefore it was necessary to investigate the localization of intracellular viral RNA-replicating structures prior to phenol extraction. These structures could be associated with cell membranes, or with ribosomes, or not associated with any recognizable cellular structures. In order to obtain meaningful data it was first necessary to obtain reproducible patterns of polyribosomes in uninfected cells. This was accomplished by adding poly-L-ornithine to the media during preparation of spheroplasts and lysate. This polyamino acid has been used to enhance titers of infectious RNA from poliovirus,⁶ presumably by protecting the viral RNA from degradation by nucleases. The authors are particularly grateful to Dr. S. Dales of this institute for suggesting the use of this compound.

Materials and Methods.—*Bacteria and virus:* *Escherichia coli* strain 3000, a nonlysogenic Hfr strain, and RNA bacteriophage R17, free of λ bacteriophage, were used in all experiments.

Media and solutions: TCGI synthetic growth medium has been described.¹ PHO: TCGI with 0.0015% casamino acids ($1/100$ normal concentration); chloramphenicol (Parke, Davis and Co.), 100 $\mu\text{g}/\text{ml}$; sodium azide, 0.005 *M*; poly-L-ornithine (Mann Research Lab., Inc.), 10 $\mu\text{g}/\text{ml}$. PH 1: Tris buffer, 0.1 *M*, pH 8.1 (5°); chloramphenicol, 100 $\mu\text{g}/\text{ml}$; poly-L-ornithine, 10 $\mu\text{g}/\text{ml}$; 20% sucrose (Mann Research Lab., Inc., ribonuclease free). TMK: Tris buffer, 0.005 *M*, pH 7.4 (5°); KCl, 0.06 *M*; chloramphenicol, 100 $\mu\text{g}/\text{ml}$; poly-L-ornithine, 10 $\mu\text{g}/\text{ml}$; sodium azide, 0.005 *M*. TMK 1: Tris buffer, 0.005 *M*, pH 7.4 (5°); KCl, 0.06 *M*; MgSO_4 , 0.01 *M*.

Preparation of labeled cell lysates from uninfected cells: The volumes refer to a starting volume of 1 liter of cells. *E. coli* was grown to a titer of $2 \times 10^8/\text{ml}$ in TCGI, harvested, and resuspended in 300 ml of prewarmed TCGI with $1/100$ casamino acids. After 5 min further incubation at 37°, the cells were labeled for 3 min with C^{14} -amino acids at 1 $\mu\text{c}/\text{ml}$. The amino acids were a mixture of 15 L-amino acids uniformly labeled at approximately 40 mc per milliatom of carbon (New England Nuclear Corp., NEC-445). In many experiments the cells were labeled for 15–20 sec with H^3 -uridine, at 1 $\mu\text{c}/\text{ml}$ (H^3 -uridine, the Radiochemical Centre, Amersham; specific activity 25.4 c/mM). Other concentrations of H^3 -uridine and other labeling times were also used and these are specified in the tables and figure legends.

The incorporation was stopped by pouring the cells over 100 ml of PHO which had been frozen at -70° . The cells were centrifuged, washed once with PH 1, and resuspended in 1.8 ml of PH 1. Lysozyme spheroplasts were then prepared at 0° by addition of lysozyme (Muramidase, $2 \times$ crystallized, Worthington Biochemical Corp.) to a final concentration of 400 $\mu\text{g}/\text{ml}$ plus 1 mM

sodium ethylenediaminetetraacetate (neutralized) to a final concentration of 0.5 mM. Spheroplast formation was always controlled by dark-field microscopy and usually was complete in 10 min. When spheroplast formation was complete, $MgSO_4$ was added to a final concentration of 0.01 M and the spheroplasts were centrifuged and resuspended in 0.7 ml of TMK for 1–2 min at 0°; after this time DNase was added to a final concentration of 1 $\mu g/ml$ and Brij 58 (Atlas Chemical Industries, Inc., Wilmington, Del.), a nonionic detergent, to a final concentration of 0.6%. From this point on, the lysate was always transferred with wide-bore pipettes to avoid shearing of the polyribosomes. The lysate was centrifuged at 10,000 g for 5 min (Sorvall GSA rotor) and the supernate was collected. Between 10 and 20 OD₂₆₀ of this supernate was used for each sucrose gradient centrifugation. Optical densities were read on a $1/100$ dilution in TMK 1.

Preparation of labeled cell lysates from infected cells: Cells were grown in TCGI to $2 \times 10^8/ml$ and then harvested by centrifugation. The cells from 1 liter of medium were resuspended in 2–2.5 ml of TCGI and infected with R17 bacteriophage by adding stock virus at an input multiplicity of 10. After an adsorption period of 3 min at room temperature, the infected cells were diluted into 300 ml of prewarmed TCGI and infection was allowed to proceed for the desired length of time. If the cells were to be labeled with both C^{14} -amino acids and H^3 -uridine, they were then centrifuged and resuspended in TCGI with $1/100$ casamino acids and treated as the uninfected cells were treated. If they were to be labeled only with H^3 -uridine, this was added directly to the cells in TCGI. This latter procedure provided a much better defined period of infection. All subsequent procedures were as described above for noninfected cells, except as described in the appropriate places in the tables and figure legends.

Preparation of nucleic acids: Nucleic acid was extracted from the polyribosome fractions with phenol-sodium dodecyl sulfate, as previously described.¹

Results and Discussion.—Characteristics of polyribosomal patterns in noninfected cells: A typical polyribosome gradient from uninfected cells is shown in Figure 1. Polysome peaks from dimers to pentamers were always resolved and occasionally a hexamer peak could be seen. Larger polyribosomes formed a continuous distribu-

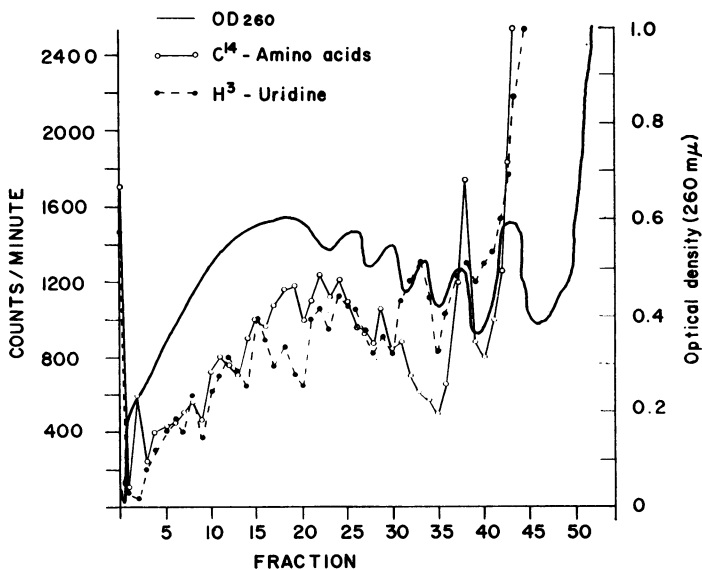


FIG. 1.—Polyribosomes from uninfected *E. coli* 3000. The cells at $6 \times 10^8/ml$ were labeled for 3 min with C^{14} -amino acids and for 1 min with H^3 -uridine. Standard polysome gradient: linear sucrose gradient (40–10% sucrose in TMK buffer, 16.2 ml total volume) centrifuged in SW 25.3 rotor, 25,000 rpm, 2°C, 3.25 hr, stopped without braking. The optical density patterns were obtained by continuous monitoring of the samples during collection of the gradient. —OD₂₆₀; ○—○ C^{14} -label; ●—● H^3 -label.

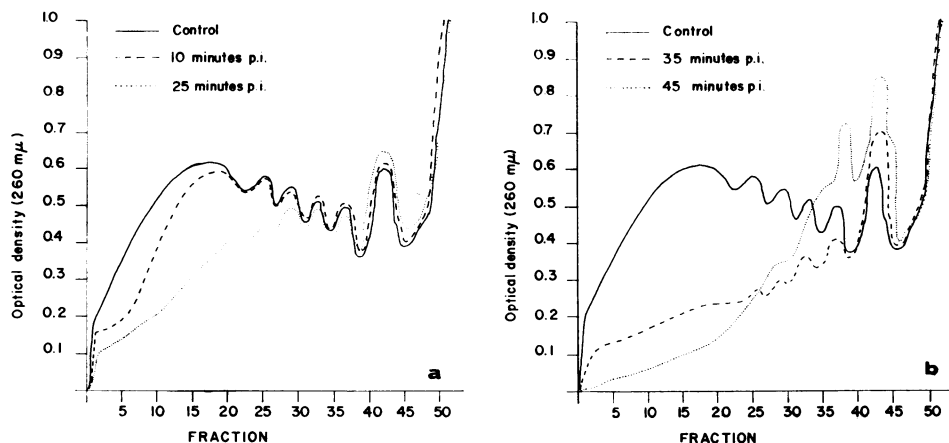


FIG. 2.—Alterations in polyribosome distributions in *E. coli* after infection with bacteriophage R17. All curves are plots of OD_{260} from standard polysome gradient.

(a) — Non infected control; - - 10 min p.i., ··· 25 min p.i.

(b) — Non infected control; - - 35 min p.i., ··· 45 min p.i.

tion under the conditions of fractionation used here. The areas under the curves were measured by planimetry. Excluding the free RNA, the polyribosomal area from dimers on up comprised 70–80 per cent of the total ribosomal region. By plotting $S^{3/2}$ versus N ,^{7, 8} where S is the sedimentation constant estimated by the method of Martin and Ames⁹ and N is the number of ribosomes per polysome, a straight line relationship was found between $N = 1$ and 6. This relationship was then used to estimate the number of polyribosomes in the larger complexes.

The distribution of nascent protein labeled with a three-minute pulse of C^{14} -amino acids followed the distribution of polyribosomes (Fig. 1). Excluding the soluble protein, approximately 7 per cent of the total C^{14} -radioactivity was found in the pellet. The one-minute pulse label of H^3 -uridine also followed the OD pattern (Fig. 1), as did a 10- to 15-second pulse of H^3 -uridine.

Characteristics of polyribosome patterns in infected cells: Already at ten minutes p.i. there was a decrease in the relative amount of polyribosomes of size greater than hexamers (Fig. 2a). By 25 minutes p.i., close to the end of the latent period, the polyribosomal distribution peaked around trimers (Fig. 2a). At later times there was a progressive decrease in polyribosomes and an increase in monomers. At 45 minutes p.i. there was a prominent peak at 79–81S, corresponding to the position of added purified H^3 -labeled bacteriophage R17.

Concomitant with the altered polyribosomal distributions were changes in the distribution of nascent protein and pulse-labeled RNA (Fig. 3a and b). At ten minutes p.i. there was no discernible difference in the pattern of label in control and infected cells. A decrease in label in the region of the larger polyribosomes was clearly evident at 25 minutes p.i. and was very striking at 45 minutes p.i. At 25 minutes p.i. only 5 per cent of the nascent protein was found in the pellet. Whereas both the dimer peak (fraction 38) and the bacteriophage peak (79–81S, at fraction 40) of C^{14} -protein were prominent at 25 minutes p.i., only the bacteriophage peak was seen at 45 minutes p.i.

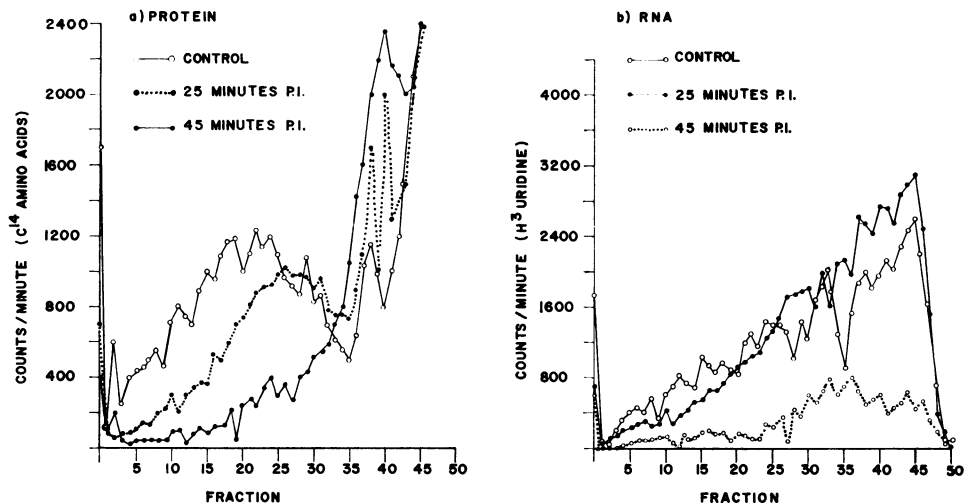


FIG. 3.—Alterations in polysome distributions of nascent protein and labeled RNA in *E. coli* after infection with bacteriophage R17. Standard polysome gradient. (a) Nascent protein, 3-min label with C^{14} -amino acids. ○—○ control; ●—● 25 min p.i.; ●—● 45 min p.i. (b) 1-min label with H^3 -uridine. ○—○ control; ●—● 25 min p.i.; ○—○ 45 min p.i.

At 25 minutes p.i. there was a decrease in the amount of labeled RNA which sedimented as heavy polyribosomes and a relative increase in the amount sedimenting in the region from dimers to the top of the gradient. The incorporation of H^3 -uridine into RNA throughout the gradient was greatly reduced after 45 minutes of infection and was almost negligible in the area of the heavy polyribosomes.

Localization of RNase-resistant RNA: The general approach was to label infected cells with a 1-minute pulse of H^3 -uridine at 30 minutes p.i. and then to divide a polysome gradient into appropriate fractions, phenol-extract each fraction, and test for RNase resistance. In the example analyzed in Table 1 the samples were pooled into 13 fractions, 5 of which were more resistant to RNase than the others (cf. Table 1).

Centrifugation in a 10–40 per cent sucrose gradient for 195 minutes at 25,000 rpm did not clearly separate the ribosomal subunits from each other or from the region containing the 70S monomers or from the soluble RNA. In order to examine this area more closely, lysates were centrifuged for eight hours at 25,000 rpm on a standard polysome gradient of 10–40 per cent sucrose. The OD_{260} pattern from lysates of uninfected cells had a 70S monomer peak which was large in relation to the polysome fractions, probably due to some degradation of the polysomes during the longer period of centrifugation employed in this experiment (Fig. 4). While polyribosomes containing as many as five to six ribosomes were still discernible, the larger polyribosomes were pelleted on the bottom of the centrifuge tube (Fig. 4). The samples from the virus-infected cell lysates were pooled into ten fractions, phenol-extracted, and the samples were examined for RNase resistance with the results shown in Table 2.

Is the RNase-resistant RNA found in the polysome region actually associated with polysomes? In a preliminary investigation of this question, the polysomes were dissociated in a low concentration of magnesium ($5 \times 10^{-5} M$) and the disso-

TABLE 1
LOCALIZATION WITHIN POLYRIBOSOMES OF RNASE-RESISTANT RNA FOLLOWING A 30-MIN INFECTION WITH R17

Gradient fraction*	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
Cellular fraction† 14-12	11	10	-9	8-7	6	5	4	3	2	70S	50S	30S	Soluble
RNase resist.‡ (%)	29 ± 2	30 ± 1	11 ± 2	10 ± 2	18 ± 1	25 ± 1	10 ± 1	10 ± 1	6 ± 3	5 ± 2	10 ± 6	2 ± 1	18 ± 1

* Cell lysates were centrifuged on a 10-40% linear sucrose gradient at 25,000 rpm for 3.25 hr at 2°C (SW 25.3 rotor). Cells were labeled with 10 µc/ml H³-uridine for 1 min. † Numbers refer to number of ribosomes in a polysome. ‡ Dimer to pentamers could be resolved as individual peaks; hexamers and higher orders of polysomes were calculated from Czer's equation. † RNase treatment: 1 µg/ml RNase at 37°C for 10 min. This data represents the average of two separate experiments.

TABLE 2
LOCALIZATION WITHIN THE RIBOSOMAL SUBUNIT REGIONS OF RNASE-RESISTANT RNA FOLLOWING 30 MIN OF INFECTION WITH R17

Gradient fraction*	I	II	III	IV	V	VI	VII	VIII	IX	X
Cellular fraction†	5-6	4	3	2	70S	50S	50-30S	30S	30S-sol.	Soluble
RNase resist. (%)‡	17 ± 2	6 ± 1	6 ± 1	5 ± 2	6 ± 1	15 ± 1	7 ± 1	7 ± 2	5 ± 1	19 ± 1

* Cell lysates centrifuged on a linear 10-40% sucrose gradient at 25,000 rpm for 8 hr at 2°C (SW 25.3 rotor). Cells were labeled with 10 µc/ml H³-uridine for 1 min. † Numbers refer to number of ribosomes in a polysome. ‡ RNase treatment: 1 µg/ml RNase incubated at 37°C for 10 min.

TABLE 3
EFFECT OF MG⁺⁺ CONCENTRATION ON THE DISTRIBUTION OF TOTAL H³-RNA AND RNASE-RESISTANT H³-RNA

Gradient fraction	Cellular components	Cpm in Each Fraction		Per cent of Total cpm		RNase-Resistant cpm in Each Fraction†	
		+ Mg*	- Mg†	+ Mg	- Mg	+ Mg	- Mg
I-IX	Polyribosomes	9 × 10 ⁶	0.1 × 10 ⁶	42.5	0.5	1.5 × 10 ⁶	0.02 × 10 ⁶
X-XII	70S-subunits	4 × 10 ⁶	12 × 10 ⁶	18.0	59.5	0.2 × 10 ⁶	2.8 × 10 ⁶
XIII	Soluble	8.5 × 10 ⁶	8 × 10 ⁶	39.5	40.0	1.5 × 10 ⁶	1.3 × 10 ⁶

Cells were infected with R17 for 30 min and labeled with 20 µc/ml H³-uridine for 1 min. Lysate centrifuged on a 40-10% sucrose gradient at 25,000 rpm for 3.25 hr 12°C (SW 25.3 rotor).
* + Mg, Mg⁺⁺ concentration of lysate and gradients was 1 × 10⁻³ M.
† - Mg, Mg⁺⁺ concentration of lysate and gradients was 5 × 10⁻³ M.
‡ Acid-insoluble cpm following incubation with 1 µg/ml of RNase at 37°C for 10 min.

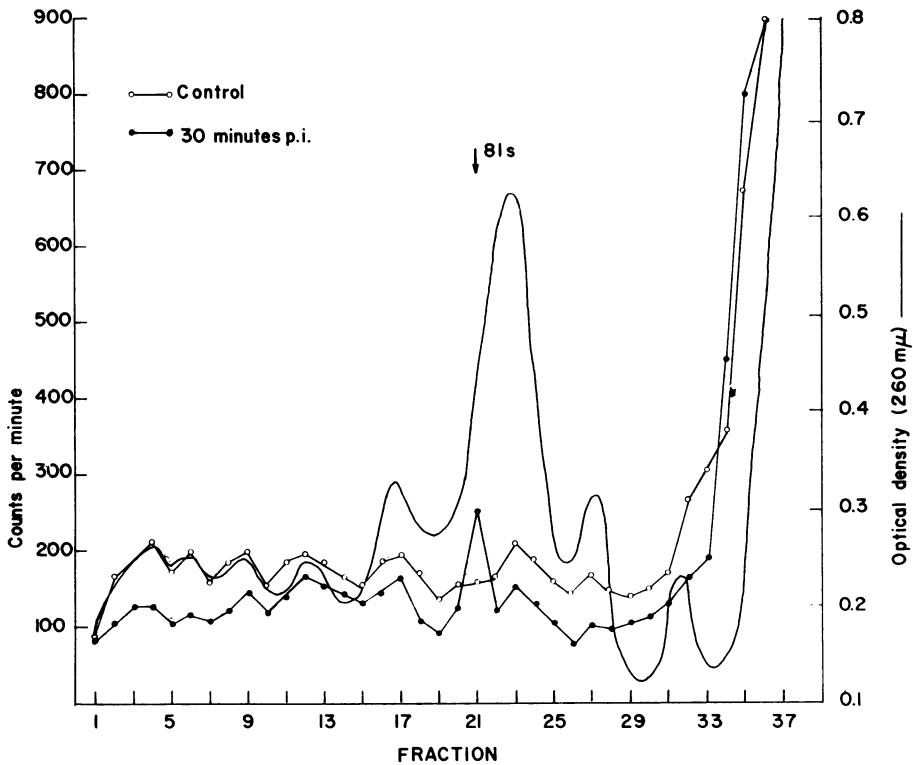


FIG. 4.—Centrifugation of *E. coli* lysates to resolve ribosomal subunits. Standard polysome sucrose gradient, centrifuged for 8 hr at 25,000 rpm. — OD₂₆₀ pattern from uninfected cell; ○—○ 1 min H³-uridine label, uninfected cell; ●—● 1 min H³-uridine label, cell infected for 30 min.

ciated polysomes were then centrifuged in the same low concentration of magnesium. The optical density patterns of both control and infected cells demonstrated the presence of 50 and 30S subunits and no 70S ribosomes. In the material from infected cells there was a peak of radioactive RNA sedimenting at about 40S and this was not present in the control patterns. The region which represented polysomes under standard conditions now contained 0.5 per cent of the radioactivity as compared with 42.5 per cent under standard conditions (Table 3). Although 20 per cent of this 0.5 per cent is still RNase-resistant, we must emphasize the disappearance of almost all of the radioactivity, RNase-resistant and -sensitive, from the polysome region. This experiment suggests that the RNase-resistant RNA found in the polysome fraction may be associated with ribosomes, and that the Mg⁺⁺ sensitivity could be due to dissociation of ribosomes from the single-stranded component of replicative intermediate at low concentrations of Mg⁺⁺.

Although the steady-state polysome distributions reported here were similar to those previously reported from many laboratories, for example, that described by Kihō and Rīch,¹⁰ some degradation of polysomes may have occurred during preparation. According to Mangiarotti and Schlesinger, the presence of 70S monomers in the gradient is indicative of some degradation of polysomes.¹¹ Nevertheless, the

comparison of polysome patterns in infected and control cells should be significant because the striking decrease in polysomes in infected cells can be correlated with a decrease in the rate of host cell RNA and protein synthesis.¹²⁻¹⁴ The localization of double-stranded RNA in the polysome fractions, as reported here and also by Godson and Sinsheimer using parental-labeled RNA,¹⁵ should also be significant since no double-stranded RNA is found in the uninfected cell. The basis for the inhibition of cellular macromolecular synthesis after infection with a RNA bacteriophage is not known and may not be essential for the replication of the bacteriophage since it does not occur in the case of bacteriophage f2.¹⁶

Summary.—Double-stranded RNA can be demonstrated to be associated with polysomes in cells infected with bacteriophage R17. Particularly prominent concentrations of double-stranded RNA are found in polysomes of size 11–14 and in the pentamer region. Some double-stranded RNA also sediments close to the 50S subunit and some is found in the region of free RNA, i.e., sedimenting at less than 30S.

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† Postdoctoral fellow of the National Institutes of Health.

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