Ribonucleic Acid Synthesis by *Escherichia coli* C3000/L after Infection by the Ribonucleic Acid Coliphage ZIK/1, and Properties of Coliphage-ZIK/1 Ribonucleic Acid

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1. A method is described for the preparation and purification of the RNA from the RNA coliphage ZIK/1. 2. Some of the physical characteristics and infective properties of coliphage-ZIK/1 RNA were examined. 3. A method is also described for examining the type and quantity of RNA synthesized after bacteriophage infection. 4. Ribosome synthesis was decreased 15 min. after bacteriophage adsorption, bacteriophage RNA was synthesized from 15 min. to 120 min. after adsorption and intracellular bacteriophages appeared 40 min. after adsorption. Cell lysis commenced 60 min. after adsorption, and was half complete 20 min. later and 90–95% complete 120 min. after adsorption. 5. Cell division continued until 40 min. after bacteriophage adsorption. 6. Bacterial ribosomes were conserved during the infective process. 7. Intracellular bacteriophage RNA has sedimentation coefficient 28 s but after cell lysis it has sedimentation coefficient 10–5 s.

Infection of *Escherichia coli* by an RNA bacteriophage has been shown to result in a net synthesis, by the host bacterium, of RNA, protein and DNA (Paranchych & Graham, 1962). The present paper describes a more detailed analysis of the RNA synthesized by *E. coli* C3000/L after infection by the RNA coliphage ZIK/1 (Bishop & Bradley, 1965) and also examines some of the properties of the bacteriophage RNA.

METHODS

Organisms. Small-scale culture procedures for *E. coli* strains and the RNA coliphage ZIK/1 were as described by Bishop & Bradley (1965). Viable counts of bacteria were determined by plating in nutrient agar. Total cell counts were made in a haemocytometer.

For mass culture of coliphage ZIK/1 a medium was prepared containing 0.45% of KH_2PO_4 , 1.05% (w/v) of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.3% of NH_4Cl , 1.5% (w/v) of tryptone (Oxo Ltd., London, S.E.1) and 3.0% (w/v) of glycerol. The pH was adjusted to 7.1 and 2.5 l. batches in 10 l. flasks were autoclaved for 30 min. at 15 lb./in.². To 2.5 l. of medium was added 100 ml. of an overnight culture of *E. coli* C3000/L and the culture was incubated at 37° with shaking until the extinction (measured in a Unicam SP.700 spectrophotometer), read against a blank of the medium alone, rose to 0.4 (equivalent to 2×10^8 bacteria/ml.). Then 30 ml. of a sterile 0.1 M-CaCl₂ solution, 20 mg. of uracil and at least 5×10^{12} bacteriophages were added and the mixture was reincubated. At the onset of lysis, 10 g. of EDTA was added, followed, when lysis was complete (as judged by extinction measurements), by 700 g. of $(\text{NH}_4)_2\text{SO}_4$. After the $(\text{NH}_4)_2\text{SO}_4$ had

dissolved, the mixture was left overnight at 4°. The precipitate, which contained 95% of the total 4.5×10^{14} plaque-forming units present in the lysate, was recovered by centrifugation at 14000g for 20 min. in a Servall model SS-4 centrifuge (rotor GSA) operated at 4°. The precipitate was homogenized with 50 ml. of TM2 buffer [0.01 M-tris-HCl buffer, pH 7.2, containing MgCl_2 (0.1 mM)] and dialysed for 18 hr. against TM2 buffer with five changes of liquid. To remove DNA present in the lysate (Bishop & Bradley, 1965) the bacteriophage suspension was incubated for 60 min. with 1 mg. of deoxyribonuclease. The bacteriophages were subsequently recovered by centrifugation for 60 min. at 77000g in the Spinco model L ultracentrifuge and suspended in TM2 buffer. About 75% of the initial titre was present as plaque-forming units in this suspension.

To purify the bacteriophages further, the procedure of Strauss & Sinsheimer (1963) was adopted. Bacteriophages from several preparations (2.3×10^{15} plaque-forming units) were mixed with CsCl (0.55 g./g. of suspension; density 1.38 ± 0.01) and centrifuged at 100000g in the Spinco model L ultracentrifuge (rotor 40) for 24 hr. The rotor was allowed to decelerate without the brake and the bacteriophages, seen as a band, were removed with a syringe. The centrifugation was repeated and the bacteriophages were collected by puncturing the base of the tube and collecting 4-drop fractions. The pooled bacteriophage fractions (4.5×10^{14} plaque-forming units) were dialysed at 4° against TM2 buffer for 18 hr. with five changes of liquid.

Extraction of coliphage-ZIK/1 RNA. The procedure adopted for the extraction of RNA from the RNA coliphage ZIK/1 was based on those of Paranchych (1963), Knolle & Kaudewitz (1962) and Kirby (1962). To 5 ml. of bacteriophage suspension in TM2 buffer (4.5×10^{14} plaque-forming units) were added 0.5 g. of bentonite, prepared according to

the procedure of Fraenkel-Conrat, Singer & Tsugita (1961), and 0.1 ml. of aq. 1% (w/v) naphthalene-1,5-disulphonate solution. The mixture was homogenized for 10 min. at 4° and then 3 ml. of cold redistilled phenol (containing 0.1% of 8-hydroxyquinoline) saturated with TM2 buffer (containing 0.2% of naphthalene-1,5-disulphonate) was added (Kirby, 1962). This mixture was homogenized at 4° for 8 min. and the resultant emulsion centrifuged at 4° for 15 min. at 6000g in an MSE Magnum refrigerated centrifuge. After the aqueous phase had been carefully removed, an additional 5 ml. of TM2 buffer containing 0.2% of naphthalene-1,5-disulphonate was added to the phenol layer and the extraction process repeated. The two aqueous phases were combined and 25 ml. of the ethanol (at -20°) was added. The mixture was left at -20° for 3 hr., after which the precipitate was recovered by centrifugation at 6000g for 15 min., dissolved in 10 ml. of 0.01 M-tris-HCl buffer, pH 7.0, and precipitated twice more by the same procedure. Finally, the RNA was dissolved in 5 ml. of 0.01 M-tris-HCl buffer, pH 7.0, and centrifuged at 75000g for 2 hr. in the Spinco model L ultracentrifuge to remove insoluble material and residual bacteriophages. The supernatant was carefully removed and recentrifuged twice more at 75000g. The resultant clear solution was found to be free of bacteriophages and contained 490 µg. of RNA/ml., less than 10 µg. of DNA/ml. and less than 10 µg. of protein/ml., as determined by the procedures described by Nisman, Fukuhara, Demailly & Genin (1962), and had extinctions 4.5, 12.5 and 4.7 at 230, 260 and 280 mµ respectively. It also contained 46 µg. of phosphorus/ml., as determined by the method of King & Wootton (1946); consequently, it was calculated that the extinction coefficient of the RNA in 0.01 M-tris-HCl buffer, pH 7.0, at 260 mµ was $8400 \pm 100/\text{g. atom of phosphorus}$. The RNA solution was stored at 4° until used.

Spheroplasts. These were prepared according to the procedure of Bishop, Roche & Nisman (1964) and by a modification of the method of Mahler & Fraser (1959). In the latter method, *E. coli* strains were grown in 500 ml. of nutrient broth [2.4% (w/v); Oxo Ltd.] until, while in the exponential phase of growth, a concentration of $5 \times 10^8/\text{ml.}$ was reached. The bacteria were harvested by centrifugation at 16000g for 10 min. in the Servall model SS-4 centrifuge, washed twice with 0.1 M-tris-HCl buffer, pH 8.0, and suspended in 200 ml. of 0.5 M-sucrose in 0.1 M-tris-HCl buffer, pH 8.0. The suspension was warmed to 37° and 2 ml. of an aqueous solution of lysozyme (1 mg./ml.) was added. The suspension was swirled for 3 min. at 37° before 1 ml. of aq. 4% (w/v) EDTA was added. After a further 10 min. of incubation the spheroplasts were harvested by centrifugation.

Spheroplasts prepared by both procedures were washed twice with 0.6 M-sucrose in 0.1 M-tris-HCl buffer, pH 7.0, and finally suspended at a concentration of $10^{10}/\text{ml.}$ in the same solution.

Spheroplast infection by coliphage-ZIK/1 RNA. Before infection, bacteriophage RNA was disaggregated to preincubation at 37° for 20 min. (Paranchych, 1963). To 1 ml. of spheroplasts was added 0.5 ml. of an RNA solution, and the mixture was incubated at 37° for 1 min. Samples were then diluted in 0.5 M-sucrose in 0.1 M-tris-HCl buffer, pH 7.0, containing spermidine (1.0 mM), and 1 ml. portions were plated with *E. coli* C3000/L by the double-layer agar plating technique (Adams, 1959), with 1 ml. of 0.8% nutrient agar containing sucrose (0.5 M) for the top layer.

Incorporation of [^{32}P]orthophosphate and [^{14}C]uracil into bacteria and coliphage ZIK/1. The incorporation of [^{32}P]orthophosphate into bacteria and coliphage ZIK/1 was similar to that described by Bishop & Bradley (1965). *E. coli* C3000/L was inoculated into 30 ml. of 2.4% (w/v) nutrient broth containing 0.5 mc of carrier-free [^{32}P]orthophosphate (The Radiochemical Centre, Amersham, Bucks.). The bacteria were incubated at 37° until a concentration of $2 \times 10^8/\text{ml.}$ (viable count) was reached, when 0.3 ml. of a sterile 0.1 M-CaCl₂ solution and 6×10^{10} RNA coliphages were added (multiplicity of infection 10). After 15 min., 0.3 ml. of a sterile 1.0 M-MgCl₂ solution was added and the infected bacteria were allowed to lyse.

Samples (0.5 ml.) were removed at intervals before and after the addition of bacteriophages and centrifuged at 15000g for 15 min. at 0° in the MSE Magnum refrigerated centrifuge (fitted with the Super-Speed attachment) to remove bacteria and bacterial debris, and 0.1 ml. of the supernatant was diluted with 9.9 ml. of TM2 buffer.

Alternatively, 3 ml. samples of the incubation mixture were disintegrated artificially by ultrasound (see below), centrifuged and diluted as before. Samples taken before the addition of MgCl₂ to the incubation mixture were made 0.01 M with respect to MgCl₂ before disintegration.

In experiments in which both [^{14}C]uracil and [^{32}P]orthophosphate were incorporated into the bacteria, the procedure was similar. *E. coli* C3000/L were inoculated into 30 ml. of nutrient broth together with 40 µC of [^{14}C]uracil (specific activity 40.6 mc/m-mole). The bacteria were grown for 3 hr. (viable count $2.6 \times 10^8/\text{ml.}$), harvested by centrifugation and washed twice with nutrient broth. They were then resuspended in 30 ml. of nutrient broth and incubated for 30 min. (viable count $5.4 \times 10^8/\text{ml.}$), when 0.3 ml. of a sterile 0.1 M-CaCl₂ solution and 1.6×10^{11} coliphages ZIK/1 were added (multiplicity of infection 10). After a further 15 min. of incubation, 0.3 ml. of 1 M-MgCl₂ and 1.0 mc of [^{32}P]orthophosphate were added. Incubation was continued until lysis occurred and samples were periodically removed and treated as described above.

Ultrasonic disintegration. Samples (3 ml.) of bacteria were disintegrated at 4° with a Dawe Soniprobe (type 1130A), setting 4, operated at 2 A. Except where indicated, the bacteria were disintegrated for 60 sec.

Sucrose-gradient sedimentation, alkaline hydrolysis and base-ratio determination by paper chromatography. All these procedures were as described by Bishop & Bradley (1965).

A sample (2 ml.) of diluted lysate was incubated at 37° with 100 µg. of deoxyribonuclease for 45 min., and then 1 ml. was sedimented on a gradient of sucrose containing TM2 or TM1 buffer [the latter is 0.01 M-tris-HCl buffer, pH 7.0, containing MgCl₂ (0.01 M)]. The gradients were fractionated and the radioactive material was determined. A sucrose-gradient profile of radioactive material isolated from an infected cell preparation is shown in Fig. 5. The major fractions of the gradient are also indicated.

At least 96% of the total acid-insoluble ^{32}P -labelled material is RNA in the 90-70s fractions from sucrose gradients of bacterial lysates prepared as described above (Bishop & Bradley, 1965). This was confirmed by incubating the 90-70s acid-insoluble radioactive material from gradients with 20 µg. of ribonuclease for 60 min. at 37°, and redetermining the residual acid-insoluble radioactive material. Provided that lysates had been preincubated with deoxyribonuclease before sedimentation, at least 97% of

the ^{32}P -labelled acid-insoluble material in the 70–20s fractions and at least 95% of the 20–0s fractions were ribonuclease-sensitive, whether they were prepared before or after bacteriophage infection. It was assumed, in the experiments described, that predominantly the 90–70s fractions contained bacteriophages, the 70–40s fractions contained 50s ribosomes, the 40–20s fractions contained 30s ribosomes and the 20–0s fractions contained transfer and other 'soluble' RNA (Bishop & Bradley, 1965).

For experiments designed to determine the base ratios of 20–0s RNA, it was desirable to preserve the 70s ribosomes and to separate the 20–0s RNA from 30s ribosomes. Lysates in 0.01 M-MgCl₂ were therefore prepared ultrasonically from infected and non-infected bacteria, labelled with ^{32}P or with ^{14}C and ^{32}P as described above, and were not diluted before treatment for 60 min. at 37° with 1 mg. of deoxyribonuclease/ml. and sedimentation in a gradient of sucrose in TM1 buffer.

Base ratios were determined on the 35–25s or 20–0s RNA fractions of sucrose gradients and are expressed as percentages of the total radioactivity present in the four ribonucleotides.

Extraction of ribosomal and total RNA, and sedimentation in a sucrose gradient. The procedures used for phenol and sodium lauryl sulphate extraction of RNA from purified ribosomes and from infected cells and sedimentation of the RNA in a gradient of sucrose were as described by Bishop *et al.* (1964). The 35–25s RNA from infected cells was used for determination of the base ratios.

Measurement of radioactivity. Acid-, ethanol- and ether-insoluble radioactive material was determined on material labelled with both ^{14}C and ^{32}P by precipitating with 2 vol. of 10% (w/v) trichloroacetic acid the 1 ml. samples of the diluted incubation mixture, or fractions from sucrose gradients. The precipitate was filtered on 2 cm. Oxoid membrane filters (Oxo Ltd.) and washed thrice with 5 ml. of 10% trichloroacetic acid, then twice with 5 ml. of ethanol-ether (2:1, v/v) and once with 5 ml. of ether. The filters were allowed to dry and, while still flat, placed in scintillation vials containing 5 ml. of scintillation fluid (Bishop & Bradley, 1965). The filters remained flat in the vials and were observed by their transparency to be water-free.

For the determination of the total ^{14}C radioactivity in bacteria before inoculation with bacteriophages, 1 ml. samples, diluted 100-fold in TM1 buffer, were filtered through 2 cm. Oxoid membrane filters and washed thrice with 5 ml. of nutrient broth containing 0.1 mg. of unlabelled uracil/ml. The filters were then washed twice with distilled water and allowed to dry in a warm current of air (40°) while being held flat. The dry filters were counted in 5 ml. of scintillation fluid.

Membrane filters were counted for at least 5 min. in a Packard Instrument Co. Tri-Carb liquid-scintillation spectrometer (model 3002). When ^{14}C and ^{32}P were to be determined simultaneously, the discriminator for the red channel was set for ^{14}C counting at 100–175 and gain at 10%, and the discriminator for the green channel was set for ^{32}P counting at 150–400 and gain at 0.94%. Under these conditions it was found, with [^{14}C]uracil or [^{32}P]orthophosphate dried on membrane filters, that 7.3% of the ^{32}P radioactivity was counted in the red channel and 0.1% of the ^{14}C radioactivity was counted in the green channel. Suitable corrections were therefore made to correct the observed counts. With these settings, the absolute counting

efficiency for ^{32}P was 37% and that for ^{14}C was 16%. No quenching of the ^{14}C spectrum was observed with the small amounts of organic material used.

In experiments in which ^{32}P was the only radioactive isotope used, the spectrometer was set as described by Bishop & Bradley (1965). All counts were corrected for background radiation. When only ^{14}C was to be counted, the discriminator was set at 50–1000 and gain at 10%.

RESULTS

Properties of coliphage-ZIK/1 RNA

Hydrogen-bonding. The reaction of coliphage-ZIK/1 RNA with 2.0% formaldehyde required 4–5 hr., and the extinction at 260 m μ increased by 20% (Fig. 1).

The melting of coliphage-ZIK/1 RNA at two salt concentrations and various temperatures was also followed by extinction measurements at 258 m μ (Fig. 2). In 0.14 M-sodium chloride in 0.01 M-tris-hydrochloric acid buffer, pH 7.0, the midpoint of melting, T_m , was 60°. This T_m was affected neither by the addition of EDTA (final concn. 0.3 mM) nor by the addition of magnesium chloride (final concn. 0.01 M). At a salt concentration of 0.02 M-sodium chloride in 1.0 mM-tris-hydrochloric acid buffer, pH 7.0, the T_m was 46°. After heating samples at 90° for 10 min., cooling and remelting, the melting at both salt concentrations was found to be reversible.

Infection of *E. coli* spheroplasts. Freshly extracted coliphage-ZIK/1 RNA was diluted in 1.0 mM-tris-hydrochloric acid buffer, pH 7.0, containing EDTA (3 mM) and allowed to infect spheroplasts prepared for various *E. coli* strains (Table 1), and the infected spheroplasts were plated with *E. coli* C3000/L. It was confirmed that the coliphage-ZIK/1 RNA would

Fig. 1. Reaction of formaldehyde with coliphage-ZIK/1 RNA. Purified coliphage-ZIK/1 RNA was suspended at a concentration of 9.8 $\mu\text{g.}/\text{ml.}$ in 0.1 M-sodium phosphate buffer, pH 7.0. Formaldehyde was added to give a final concentration of 2% (v/v) and the reaction followed in a Unicam spectrophotometer with a sealed 1 cm. silica cell whose temperature was maintained at 37° by a cell holder in which water circulated. The relative increase in extinction at 260 m μ (0.249 at zero time) was followed during 21 hr. of incubation.

infect spheroplasts from bacteria normally resistant (WF⁻) or that had been isolated as a resistant mutant (C3000/RF) to the bacteriophage (Bishop & Bradley, 1965). This property has also been observed by Knolle & Kaudewitz (1962), Fouace & Huppert (1962) and Paranchych (1963). It was also found that, provided the infected spheroplasts were diluted 20-fold, a linear relationship existed between the amount of RNA used to infect the spheroplasts and the number of plaques obtained. It was necessary to dilute the incubation mixture to decrease the number of residual host-strain bacteria, as the efficiency of spheroplast formation by the lysozyme-EDTA method was never more than 80–90% (as determined by viable counts). Plating infected spheroplasts from strain C3000/RF or WF⁻ on the host bacterium did not give rise to plaques.

Spheroplasts prepared by the penicillin procedure

(99.99% efficiency of spheroplast formation) were not as efficiently infected by coliphage-ZIK/1 RNA as those prepared by the lysozyme-EDTA method. When 10 $\mu\text{g.}$ of preincubated coliphage-ZIK/1 RNA was incubated with 10^{10} penicillin-produced *E. coli* C3000/L spheroplasts under standard conditions, the plaque count obtained on plating with *E. coli* C3000/L was 4080 (cf. Table 1).

When 10 $\mu\text{g.}$ of coliphage-ZIK/1 RNA was suspended in 0.5 ml. of 0.5 M-sucrose in 1.0 M-tris-hydrochloric acid buffer, pH 7.0, containing EDTA (3 mM), and allowed to infect lysozyme-produced *E. coli* C3000/L spheroplasts, as described above, the plaque yield after infection was 40 (cf. Table 1). This confirms the observation by Engelhardt & Zinder (1964) that the RNA is taken up by the protoplasts simultaneously with the osmotic shock produced in the protoplasts when the RNA solution is added.

Sedimentation of coliphage-ZIK/1 RNA in a sucrose gradient. To obtain an approximate estimate of the sedimentation coefficient of coliphage-ZIK/1 RNA, 40 $\mu\text{g.}$ of the RNA in 0.1 M-tris-hydrochloric acid buffer, pH 7.0, was sedimented in a gradient of sucrose (20–5%, w/v) for 11 hr. at $90\,000g_{\text{max.}}$, fractionated and the RNA determined in each fraction from the extinction at $260m\mu$. A similar gradient was run containing 50 $\mu\text{g.}$ of purified ribosomal RNA. After sedimentation and fractionation the 23s and 16s ribosomal peaks were identified from the extinction at $260m\mu$. By comparison with the relative positions of the 23s and 16s RNA, the coliphage-ZIK/1 RNA was found to have sedimentation coefficient about $28 \pm 3\text{s}$.

Stability of ribosomes to ultrasonic disintegration

Before investigating the RNA synthesized by a bacterium after RNA-coliphage infection, a method

Fig. 2. Melting properties of coliphage-ZIK/1 RNA. The melting of purified coliphage-ZIK/1 RNA in 0.14 M-NaCl in 0.01 M-tris-HCl buffer, pH 7.0 (O), or 0.02 M-NaCl in 1.0 M-tris-HCl buffer, pH 7.0 (●), at a concentration of 9.8 $\mu\text{g./ml.}$, was followed in a Unicam spectrophotometer with a sealed 1 cm. silica cell whose temperature was regulated by a cell holder in which water circulated. The relative increase in extinction at $258m\mu$ was determined at various temperatures.

Table 1. *Infection of E. coli spheroplasts by coliphage-ZIK/1 RNA*

A 0.5 ml. sample of preincubated coliphage-ZIK/1 RNA preparation in 1.0 M-tris-HCl buffer, pH 7.0, containing EDTA (3 mM) was added to 1 ml. of spheroplasts, prepared by the lysozyme-EDTA procedure, incubated for 1 min. at 37° , diluted and homogenized with 18.5 ml. of 0.6 M-sucrose in 0.01 M-tris-HCl buffer, pH 7.0, containing spermidine (1 mM), and 1 ml. was plated with *E. coli* C3000/L, with 0.8% nutrient agar containing sucrose (0.6 M). Controls of coliphage-ZIK/1 RNA incubated similarly with bacteria alone were also run. Plaque counts were corrected for the dilution.

<i>E. coli</i> strain	Type	RNA added ($\mu\text{g.}$)	...	Plaque count			
				Spheroplast infection			Bacterial infection
				10	5	1	10
C3000/L	F ⁺			5200	2800	560	0
C3000/RF	F ⁺			5000	2800	480	0
K 10	Hfr			5160	2720	520	0
40	Hfr			4880	2460	520	0
WF ⁻	F ⁻			4680	2420	480	0

Fig. 3. Effect of ultrasonic treatment on bacteria and [2-¹⁴C]uracil-labelled material. The methods of ultrasonic disintegration, determination of the number of viable bacteria (●) and of acid-, ethanol- and ether-insoluble radioactive material (O) are described in the Methods section. Bacteria were grown in nutrient broth containing [2-¹⁴C]uracil for 3 hr., washed and recultured for 30 min. in nutrient broth alone; MgCl₂ was added (final concn. 0.01 M) and 3 ml. portions were disintegrated for various times as described. The logarithms of the percentages of the initial values are plotted.

of disintegrating bacteria had to be devised that was quick, reproducible and did not injure the main RNA fractions. The procedure chosen was that of ultrasonic disintegration. Before disintegration, 88% of the total acid-insoluble ¹⁴C radioactivity was rendered soluble by incubation in TM1 buffer at 37° for 60 min. with 100 μg. of ribonuclease, and 6% was rendered soluble by incubation with 100 μg. of deoxyribonuclease. Fig. 3 shows the fate of the ¹⁴C-labelled material at various times of ultrasonic disintegration in the presence of 0.01 M-magnesium chloride and also shows the concomitant loss of bacterial viability. After counting the remaining radioactivity, the membrane filters were incubated at 37° for 60 min. with 100 μg. of ribonuclease with or without 100 μg. of deoxyribonuclease in 1 ml. of TM2 buffer. After incubation, the residual radioactivity was redetermined. At least 99% of the counts remaining after disintegration was rendered soluble by both nucleases, whereas 95–99% of the counts after 0–40 sec. disintegration and at least 99% of the counts after 50–120 sec. disintegration were rendered soluble by ribonuclease alone. One-tenth of the total counts was rendered soluble during the first 40 sec. of disintegration but this amount did not change appreciably during the subsequent 80 sec. of disintegration.

These experiments showed that at least 90% of the ¹⁴C-labelled material was RNA and at least 6% DNA. Ultrasonic treatment selectively disintegrated DNA and perhaps a small RNA fraction. Whether this small RNA fraction was of ribosomal origin could not be determined. However, as most of the cell RNA is present in ribosomes it was apparent that the bacterial ribosomes were stable to the ultrasonic treatment used under the conditions of these experiments. This was confirmed during the analysis of cell RNA liberated from infected bacteria.

Analysis of the RNA synthesized after bacteriophage infection

To separate 80s bacteriophages from *E. coli* ribosomes present in a cell lysate, advantage was taken of the fact that, in 0.2 mM- and 0.1 mM-magnesium chloride, 70s ribosomes dissociate into 50s and 30s particles (Tissières, Watson, Schlessinger & Hollingworth, 1959; Bishop & Bradley, 1965).

The fate and type of RNA synthesized before and after bacteriophage adsorption was examined by disintegrating with ultrasound in the presence of 0.01 M-magnesium chloride, infected and non-infected bacteria (grown in [³²P]orthophosphate), removing bacterial debris by centrifugation, diluting the supernatant in 0.1 mM-magnesium chloride, incubating with deoxyribonuclease and sedimenting in a sucrose gradient. The total amounts of radioactivity (present as RNA; see the Methods section) in the 90–70s fractions (containing bacteriophages), 70–40s fractions (containing 50s ribosomes), 40–20s fractions (containing 30s ribosomes) and 20–0s fractions (containing transfer and other RNA) were determined after the gradient had been fractionated (continuous lines in Fig. 4). On the basis of the ³²P content the ratio of 70–40s to 40–20s material present after diluting the supernatant of ultrasonically disrupted uninfected bacteria with TM2 buffer was about 2:1. Although no correction was made for the overlap of particles in the gradient, this ratio agrees reasonably well with that found for 50s and 30s particles from purified 70s particles (i.e. 2.5:1; Tissières *et al.* 1959). The uncorrected ratio for 70–40s to 40–20s ribosomes, namely 2:1, was also found for bacterial ribosomes liberated by lysing bacteria after bacteriophage infection. It was assumed therefore that ultrasonic disintegration of bacteria did not substantially alter the ribosome composition. The liberation, by lysing bacteria, of ribosomes, viable bacteriophages and other RNA was also examined by a similar procedure except that the culture was not treated with ultrasound (broken lines in Fig. 4).

Ribosome synthesis was exponential in the period preceding bacteriophage infection and continued at

Fig. 4. Analysis of the synthesis of bacteriophages and RNA before and during bacteriophage infection. Bacteria were grown for 3 hr. in the presence of [32 P]orthophosphate, treated at zero time with bacteriophages (multiplicity of infection 10) and CaCl_2 and 15 min. later with MgCl_2 (final concn. 0.01 M) and allowed to lyse. Samples were removed periodically and the extinction at 600 mμ and total cell count were determined. Bacteria and bacterial debris were removed by centrifugation and the supernatant was diluted 100-fold with TM2 buffer. To determine the total bacteriophage count and analyse the total ^{32}P -labelled RNA, duplicate samples were treated with ultrasound before the removal of bacterial debris. The samples in TM2 buffer were incubated for 45 min. at 37° with 50 μg. of deoxyribonuclease/ml. and then 1 ml. samples were sedimented on a gradient of sucrose. Fractionation of the gradient, determination of the acid-insoluble ^{32}P -labelled material and bacteriophage content of the supernatant before dilution in TM2 buffer were as described by Bishop & Bradley (1965). (a) Extinction: infected (▽) and non-infected culture (▼); total cell count: infected (□) and non-infected culture (■). (b) ^{32}P radioactivity of 70–40s fractions: total (●) and extracellular (○); ^{32}P radioactivity of 40–20s fractions: total (■) and extracellular (□). (c) ^{32}P radioactivity of 90–70s fractions: total (▲) and extracellular (Δ); ^{32}P radioactivity of 20–0s fractions: total (●) and extracellular (○).

the same rate until 10–15 min. after infection, when the rate of ^{32}P incorporation into ribosomes decreased substantially and stopped by 100 min. after infection. Bacterial lysis began 60 min. after infection, as judged by the liberation of 50s or 30s ribosomes and the liberation of viable or ^{32}P -labelled bacteriophages or 20–0s RNA into the culture medium. There was also a fall in total cell count and extinction at 600 mμ. Lysis was about 50% complete 20 min. later and 90–95% complete 120 min. after infection. The intracellular appearance of viable and ^{32}P -labelled bacteriophages commenced about 40 min. after infection. At 80 min. after infection, when 50% of the bacteria had lysed, the synthesis of viable bacteriophages was 33% complete (by comparison with the total number of viable or ^{32}P -labelled bacteriophages present in the artificially or naturally lysed culture at 120 or 200 min. after infection). This suggested that the bacterium for which lysis is delayed, for one reason or another, liberates more bacteriophages/cell than those in which lysis occurs earlier.

The incorporation of ^{32}P into 20–0s RNA was exponential from before infection until 10–15 min. after infection, when the rate decreased and maintained a decreased rate until about 120 min. after infection. However, the decreased rate of synthesis of 20–0s RNA from 10–15 min. after infection was substantially higher than the incorporation of ^{32}P into ribosomes in the same period preceding infection (Fig. 4). Most of the ^{32}P incorporated into the 20–0s fraction was sedimented in the 10–5s fraction.

The extinction and total cell count of the incubation mixture during infection is also given in Fig. 4, together with those of a control non-infected culture. Cell division continued until 40 min. after adsorption, and the extinction increased until 60 min. after adsorption.

The base ratios ($\pm$ S.E.M.) of the 20–0s RNA fractions from artificially broken uninfected bacteria (in 0.01 M-magnesium chloride), calculated from 15 analyses of fractions from three separate gradients, were 28.8 ($\pm$ 0.4), 21.0 ($\pm$ 0.4), 15.5 ($\pm$ 0.3) and 34.7 ($\pm$ 0.3) for cytosine, adenine, uracil and guanine respectively. The base ratios ($\pm$ S.E.M.) of the 20–0s RNA fractions from lysates of infected bacteria (in 0.01 M-magnesium chloride), calculated from 17 analyses of fractions from three separate gradients, were 25.3 ($\pm$ 0.4), 23.2 ($\pm$ 0.4), 25.7 ($\pm$ 0.3) and 25.8 ($\pm$ 0.3) for cytosine, adenine, uracil and guanine respectively. Though it was apparent that the base ratios for 20–0s RNA from lysates of infected bacteria differed from the base ratios of RNA isolated from uninfected bacteria, it was also observed that they were dissimilar to coliphage-ZIK/1 RNA (Bishop & Bradley, 1965). However, this could be due to the fact that the bacteria were grown in the presence of [^{32}P]orthophosphate before

infection and contained 20-0s RNA having the base ratios given above.

Another experiment was therefore devised in which [^{32}P]orthophosphate was added to the culture 15 min. after infection, and, to investigate whether ribosomes were conserved or broken down during bacteriophage infection, the cells were labelled with [^{14}C]uracil before infection. Bacteria were inoculated with [^{14}C]uracil and grown for 3 hr., washed and then recultured for one generation. The acid-soluble pool of [^{14}C]uracil (the difference between the total ^{14}C incorporated into bacteria and the ^{14}C incorporated into acid-insoluble material) was 5% of the total incorporation before the bacteria were removed from the medium containing [^{14}C]uracil and less than 1% after the bacteria had grown for one generation in the [^{14}C]uracil-free medium. The bacteria were then inoculated with bacteriophages and calcium chloride, and 15 min. later 1.0 mc of [^{32}P]orthophosphate was

added. Incubation was continued until lysis was complete.

The total ^{14}C incorporated into the 90-70s, 70-40s, 40-20s and 20-0s RNA fractions was, within experimental error, constant from 20 min. before to 200 min. after infection. The percentages ($\pm$ s.e.m.) of the total acid-insoluble ^{14}C -labelled material recovered from a gradient were 4 ($\pm$ 2), 46 ($\pm$ 2), 23 ($\pm$ 1) and 21 ($\pm$ 1) for artificially lysed infected and non-infected bacteria from 20 min. before to 200 min. after infection, for 90-70s, 70-40s, 40-20s and 20-0s RNA fractions respectively (Fig. 5). For extracellular RNA the percentages were 4 ($\pm$ 2), 43 ($\pm$ 2), 21 ($\pm$ 1) and 23 ($\pm$ 2) for the same fractions from 60 to 200 min. after infection. In view of the observation that the amount of ^{14}C present in the 90-70s region was similar for samples of uninfected and infected bacteria, and that a small overlap of the 50s material occurs in the 90-70s region (Bishop & Bradley, 1965), it was apparent that the incorporation of ^{14}C -labelled material into bacteriophages was negligible.

The incorporation of ^{32}P into 90-70s, 70-40s, 40-20s and 20-0s RNA is shown in Fig. 6. It was observed that incorporation into 70-40s, 40-20s and 20-0s RNA from 15 min. to 50 min. after infection was linear. The incorporation of ^{32}P into bacteriophages commenced about 40 min. after infection and was not linear (Fig. 6).

Fig. 5. Sucrose-gradient sedimentation of ^{32}P - and ^{14}C -labelled material from infected cells. *E. coli* C3000/L was grown in the presence of [^{14}C]uracil for 3 hr., centrifuged, washed and grown for one generation without [^{14}C]uracil. The culture was then treated with the RNA coliphage ZIK/1 and CaCl_2 was added. After incubation for 15 min at 37° , [^{32}P]orthophosphate and MgCl_2 were added (see the Methods section). A sample was removed 75 min. later, disintegrated ultrasonically and bacterial debris removed by centrifugation. The supernatant was diluted 100-fold with TM2 buffer, treated with deoxyribonuclease and 1 ml. sedimented on a 20-5% (w/v) gradient of sucrose in TM2 buffer for $3\frac{1}{2}$ hr. The gradient was fractionated and the acid-insoluble radioactive material determined as described in the Methods section: ^{14}C -labelled material ($\bullet$); ^{32}P -labelled material ($\circ$).

Fig. 6. Analysis of the ^{32}P -labelled RNA synthesized during RNA-coliphage infection. Bacteria were grown for 3 hr. in nutrient broth containing [^{14}C]uracil, then washed and recultured for 30 min. in uracil-free medium. Bacteriophages and CaCl_2 were added at zero time and 15 min. later MgCl_2 and [^{32}P]orthophosphate were added. Samples were removed periodically and treated with ultrasound, and the ^{32}P -labelled material was analysed as described in Fig. 4 except that the acid-, ethanol- and ether-insoluble ^{32}P radioactivity was determined and differentiated from ^{14}C radioactivity, as described in the Methods section: 90-70s fractions (broken line, $\bullet$); 70-40s fractions ($\blacktriangle$); 40-20s fractions ($\triangle$); 20-0s fractions ($\circ$).

Fig. 7. Sucrose-gradient sedimentation of RNA extracted from infected bacteria. Bacteria were labelled and infected as described in Fig. 5. RNA was extracted 60 min. after the addition of the bacteriophages by the sodium lauryl sulphate-phenol method and DNA removed by treatment with deoxyribonuclease (Bishop *et al.* 1964). Sedimentation on a 20–5% (w/v) sucrose gradient was as described by Bishop *et al.* (1964). The acid-insoluble radioactive material was determined after fractionation of the gradient (see the Methods section): ^{14}C -labelled material (●); ^{32}P -labelled material (○).

The base ratios of the 20–0s RNA were also determined in the experiment in which [^{32}P]orthophosphate was added 15 min. after bacteriophage infection. Samples from cultures lysed by infection in the presence of 0.01 M-magnesium chloride were sedimented in a gradient of sucrose in TM1 buffer, fractionated and the 20–0s fractions analysed. The base ratios ($\pm$ S.E.M.) of the 20–0s RNA, calculated from 15 analyses, were 24.2 ($\pm$ 0.4), 23.5 ($\pm$ 0.4), 27.9 ($\pm$ 0.3) and 24.4 ($\pm$ 0.3) for cytosine, adenine, uracil and guanine respectively. These base ratios are, within experimental error, similar to those obtained by Bishop & Bradley (1965) for coliphage-ZIK/1 RNA.

To determine whether, before lysis, the intracellular bacteriophage RNA had sedimentation coefficient 10–5s, the total RNA was extracted, with sodium lauryl sulphate, from infected cells 60 min. after bacteriophage adsorption. The cells had been labelled before infection with [^{14}C]uracil, grown for one generation without [^{14}C]uracil, infected with bacteriophage and 15 min. later [^{32}P]orthophosphate added (see the Methods section). When sedimented in a gradient of sucrose (Fig. 7), 80% of the total ^{32}P -labelled RNA sedimented in the 28s region (calculated after correction for the 23s ribosomal RNA) and had base ratios similar to those of coliphage-ZIK/1 RNA. The ribosomal RNA sedimented at 23 and 16s and was labelled by both ^{14}C and ^{32}P . The amount of ^{32}P -labelled 10–5s RNA was about 8% of the total ^{32}P label.

The extracellular 10–5s RNA found in lysates of

ultrasonically disintegrated cells or cells lysed after infection was therefore a result of lysis and mostly originated from intracellular 28s RNA.

Cell lysis in the absence of magnesium chloride

The purification procedure originally used for RNA bacteriophages (Bishop & Bradley, 1965) might not be expected to remove ribosomes from the bacteriophages. However, purified bacteriophage preparations contained very few ribosomes. To examine this point, bacteria were grown in [^{32}P]orthophosphate, infected and allowed to lyse as described above except that no magnesium chloride was added to the culture. The recovery and incorporation of ^{32}P into 70–40s and 40–20s RNA from artificially lysed cultures was similar to that shown in Fig. 6 until 60 min. after bacteriophage adsorption, when the recovery of ^{32}P -labelled material in these fractions began to decrease with time. At 80 min. after adsorption it was 50% of the 60 min. value and at 120 min. and 200 min. it was 10% of the 60 min. value. The maximum 70–40s and 40–20s RNA recovered for naturally lysed cultures was never more than 10% of the 60 min. value obtained from artificially lysed cells. The recovery and incorporation of ^{32}P into 90–70s and 20–0s RNA throughout the whole infective process was similar to that shown in Fig. 6. Therefore, when cell lysis was allowed to proceed in the absence of 0.01 M-magnesium chloride, 90% of the cell ribosomes were broken down.

DISCUSSION

Properties of coliphage-ZIK/1 RNA. Comparison of coliphage-ZIK/1 RNA with coliphage-MS2 RNA (Strauss & Sinsheimer, 1963) shows that there are certain similarities: the sedimentation coefficient, extinction coefficient, reaction with formaldehyde and reversible melting properties. However, the extent and time of reaction with formaldehyde, 4–5 hr. for coliphage-ZIK/1 RNA as opposed to 12–14 hr. for coliphage-MS2 RNA, the different T_m values and relative melting of coliphage-ZIK/1 RNA confirm the previous observation that coliphage-ZIK/1 RNA has different properties (Bishop & Bradley, 1965). Fresco (1963) has postulated that the T_m of single-stranded RNA is directly related to the statistical probability of its containing guanine-cytosine pairs, inversely related to the statistical probability of its containing adenine-uracil pairs, but not related to any other base pairs. By comparison with the T_m of various RNA samples (Fresco, 1963), coliphage-ZIK/1 RNA and coliphage-MS2 RNA have T_m values close to that predicted from their guanine-cytosine contents. It seems probable therefore that the differences be-

tween coliphage-ZIK/1 RNA and coliphage-MS2 RNA in the degree of hydrogen-bonding, and therefore secondary structure, is related to their guanine-cytosine contents.

The infectivity of coliphage-ZIK/1 RNA, if related to the original number of bacteriophages from which it was extracted, is about 9×10^{-7} , which is similar to that obtained by Knolle & Kaudeqitz (1962), Fouace & Huppert (1962) and Paranchych (1963) for RNA preparations from other RNA coliphages.

Analysis of RNA synthesized after bacteriophage infection. It has been observed that bacterial ribosomes are conserved during bacteriophage infection, and that the rate of ribosome synthesis in the culture decreases shortly after bacteriophage adsorption but is maintained during the infection. Also, the 20-0s RNA synthesized from 10-15 min. after adsorption until cell lysis occurs is almost wholly bacteriophage RNA, but, unlike the intracellular bacteriophage RNA, the liberated bacteriophage RNA has sedimentation coefficient 10-5s. Recent studies with chloramphenicol and streptomycin have shown that, during the period immediately after an RNA bacteriophage adsorption, protein is synthesized that is vital for the subsequent bacteriophage-RNA synthesis (Brock, 1962; Cooper & Zinder, 1963; Paranchych & Ellis, 1964; Brownstein, 1964). Lodish, Cooper & Zinder (1964) suggested that this protein is the RNA-primed RNA polymerase, or RNA replicase that is induced by and replicates bacteriophage RNA (Weissmann, Simon & Ochoa, 1963; Haruna, Nozu, Ohtaka & Spiegelman, 1963). Until this enzyme is synthesized there is little or no bacteriophage RNA synthesized. This could account for the 10-15 min. delay in the decrease of ribosome synthesis that has been observed in the present work.

By comparing the relative ^{32}P incorporation into 20-0s and 90-70s bacteriophage RNA, about one-third of the bacteriophage RNA that is synthesized becomes incorporated into bacteriophage particles (Figs. 4 and 6). In one experiment there was, however, eight times as much bacteriophage RNA synthesized during the period from 15 to 200 min. after adsorption as ribosomal RNA during the same period, when the latter is defined by the ^{32}P incorporation into 50s and 30s particles (Fig. 4). In another experiment there was about five times as much bacteriophage RNA synthesized (Fig. 6). Neither of these calculations, however, takes into consideration a possibility that ribosomal RNA could be synthesized and broken down before incorporation into particles.

The only estimate that can be made of the amount of transfer and other non-bacteriophage low-molecular-weight RNA that is synthesized during the infective process comes from the ^{32}P -labelled RNA

extracted by sodium lauryl sulphate. At 60 min. after adsorption the ^{32}P incorporated into 10-0s RNA was about 8% of the total ^{32}P incorporation into RNA. It is not known, however, how much of this RNA is bacteriophage RNA, and consequently an estimate of 8% represents an upper limit.

The intracellular 28s RNA has, after artificial and phage-induced lysis, sedimentation coefficient 10-5s. Bacteriophage RNA, when extracted after breaking cells in a French press, also has a low sedimentation coefficient (Ellis & Paranchych, 1963).

Cell division continues after ribosome synthesis is decreased (Fig. 4). By comparison of the ^{32}P radioactivity/cell, at 60 min. after bacteriophage adsorption, the bacteria contain about 40% fewer ribosomes/cell than they did at 10 min. after adsorption (Fig. 4). In terms of total RNA, by comparison with cells 10 min. after adsorption, the cells at 40 min. and 60 min. contain respectively about 20% and 10% less RNA/cell respectively, but at 80 min. they contain 10% more RNA/cell, when, in the latter case, the maximum cell concentration reached by the culture is used for the calculation. Since, however, the cell count falls by 50% between 60 and 80 min. after adsorption, it appears that more bacteriophage RNA as well as whole bacteriophages is synthesized/cell in cells in which lysis is delayed.

Between 40 and 60 min. after adsorption, the cell count/ml. remains constant although the extinction at 600 m μ increases, suggesting that the cell volume increases during this period.

It has recently been shown that early during infection of *E. coli* by an RNA coliphage a ribonuclease-resistant double-stranded RNA is formed with sedimentation coefficient 12-16s (Weissmann & Borst, 1963; Weissmann, Borst, Burdon, Billeter & Ochoa, 1964; Kaerner & Hoffmann-Berling, 1964; Nonoyama & Ikeda, 1964). This double-stranded RNA has been demonstrated to be an intermediate in the replication of the single-stranded coliphage RNA and is composed of the parental coliphage RNA and a newly-synthesized complementary RNA strand. The decrease in the amount of parental coliphage RNA in the double-stranded replicative form that subsequently occurs confirms studies *in vitro* with the coliphage-induced RNA-primed RNA polymerase, that replication involves an asymmetric semi-conservative mechanism whereby the parental RNA strand becomes replaced by an identical newly synthesized RNA strand (Weissmann *et al.* 1963).

The amount of double-stranded RNA that is synthesized by the end of the infective process has been shown to be no more than 2-3% of the total RNA (Kaerner & Hoffmann-Berling, 1964; Weissmann *et al.* 1964). Although a double-stranded RNA has not been demonstrated in the bulk experiments reported in the present paper, a double-stranded

RNA has been found during infection of *E. coli* C3000/L by the RNA coliphage ZIK/1 (D. H. L. Bishop, unpublished work).

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