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# Determination of Base Ratios of Six Ribonucleic Acid Bacteriophages Specific to *Escherichia coli*

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1. A method is described for the isolation of single-stranded-RNA coliphages. Two of the six RNA coliphages investigated were new strains. 2. The base ratios of six RNA coliphages were determined by labelling the host bacterium with [$^{32}$P]-phosphate, purification of the radioactive coliphages and separation of 2′,3′-ribonucleotides liberated by alkaline hydrolysis of the coliphage RNA. 3. All six of the coliphages were morphologically similar, contained single-stranded RNA, and had sedimentation coefficient $80 \pm 5$S. 4. The six RNA coliphages fell into two distinct groups, both serologically and in terms of their RNA base ratios.

Several single-stranded-RNA bacteriophages have recently been isolated that are specific to donor strains ($F^+$ and Hfr) of *Escherichia coli* (Loeb & Zinder, 1961; Davis, Strauss & Sinsheimer, 1961; Paranchych & Graham, 1962; Marvin & Hoffmann-Berling, 1963). The present paper describes the isolation and purification of six RNA bacteriophages specific to *E. coli* and the determination of their base ratios.

## METHODS

*Bacteria.* A number of Hfr and $F^+$ strains of *E. coli* were used to determine a suitable host for the isolation, purification and growth of RNA coliphages (Bradley, 1964). The suffixes (Table 1) /L, /F or /R to an *E. coli* strain indicate that they are mutants resistant to SBL, filamentous or RNA coliphages respectively (Bradley, 1964). The SBL coliphages are a group of *E. coli* bacteriophages having small heads and long tails. They are morphologically similar to the SBL type of *Salmonella* phage described by Bradley & Kay (1960). (For a discussion of coliphage morphological types, see Bradley, 1964.) Table 1 gives the results of measuring the efficiency of plating six RNA coliphages on the available *E. coli* strains. Bacteria from a nutrient–agar slope were inoculated into 10ml. of 2·6% (w/v) nutrient broth (Oxo Ltd., London) and incubated with shaking (70 oscillations/min.) at 37° for at least 4hr. until the bacteria had reached the exponential phase and a concentration of $2 \times 10^8$ bacteria/ml. Then 1ml. of a phage suspension was added to 1ml. of bacteria and plated by the double-layer agar method (Adams, 1959, p. 450). The plates were incubated at 37° for 12hr. and the efficiency of plaque formation was calculated by using the host *E. coli* 40 as a basis of comparison (Table 1). The number of plaques did not increase on prolonged incubation and was not affected by the amount of bacteria used for plating. Duplicate experiments gave similar results. It was decided to use as host for the isolation, purification and growth of RNA coliphages the $F^+$ *E. coli* strain C3000/L, on account of its resistance to the common and virulent SBL coliphages (Bradley, 1963) as well as its high efficiency as host to RNA coliphages.

The bacteria were maintained on nutrient–agar slopes.

*RNA coliphages.* The methods of growth, purification and preparation of titres of RNA coliphages up to $10^{11}$ plaque-forming units/ml. were as described by Bradley (1963). For all six RNA coliphages (ZIK/1, ZG, ZJ/1, $\alpha$15, ZS/3 and ZL/3), the plaque-forming units were assayed by the double-layer agar plating technique. The final concentration of nutrient broth (Oxo Ltd.) in the agar layers was 2·6% (w/v) in the lower 1·2% (w/v) agar no. 3 (Oxo Ltd.) and 2·0% (w/v) in the upper 0·4% agar layer. These concentrations of nutrient broth were found to be optimum for plaque formation. Plates were incubated at 37° for 12hr.

The isolation of three of the RNA coliphages (ZIK/1, ZG and ZJ/1) and of the filamentous (ZG2) and $\phi$X-type (S1) coliphages was as described by Bradley (1964). The RNA coliphage $\alpha$15 was isolated from Braid Burn, Edinburgh, by the method of Bradley (1964). The RNA coliphages ZS/3 and ZL/3 were newly isolated by an alternative procedure similar to that described by Paranchych & Graham (1962), use being made of the observation that plaque formation by a single-stranded-RNA coliphage is inhibited by ribonuclease (Loeb & Zinder, 1961).

Sewage samples from Linlithgow and Stoke-on-Trent were shaken for 10min. with an equal volume of chloroform. The aqueous phase was centrifuged at 4000*g* for 15min. to remove large debris. Then 1ml. of the resulting supernatant was placed on top of a 24ml. linear gradient of sucrose (20–5%, w/v) prepared in 0·01M-tris buffer, pH 7·2, containing $MgCl_2$ (0·01M) (TM1 buffer). Centrifuging in the Spinco model L ultracentrifuge (rotor SW25-1) was at 25000rev/min. ($90000 g_{max.}$) for 3hr. at 0°. The base of the tube was pierced and 20-drop fractions (about 2ml.) were collected. Each fraction was dispersed by pipetting through a wide-bore pipette and divided, and 0·1mg. of ribonuclease (Sigma Chemical Co., St Louis, Mo., U.S.A.) added to one part. The number of plaque-forming units

Table 1. *Efficiency of plating eight coliphages on various strains of* Escherichia coli

The methods of measuring the efficiency of plating are described in the text. The numbers of plaques obtained were used to determine the efficiency of plating, with the host *E. coli* 40 as a basis of comparison. Plaque size is indicated by : (l), large (about 3mm.); (vl), very large (5mm. or more); (s), small (less than 2mm.). Coliphage S1 is a single-stranded-DNA $\phi$X 174-type coliphage; ZJ2 is a filamentous coliphage; ZIK/1, ZJ/1, $\alpha$15, ZL/3, ZS/3 and ZG are RNA coliphages. Coliphage-resistant bacterial mutants are designated: /F, resistant to filamentous coliphages; /R, resistant to RNA coliphages; /L, resistant to SBL coliphages (see the text).

| | | No. of plaques/no. of plaques with *E. coli* 40 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Coliphage ... | ZIK/1 | ZJ/1 | $\alpha$15 | ZL/3 | ZS/3 | ZG | S1 | ZJ/2 |
| *E. coli* strain | Type | | | | | | | | |
| C3000 | F+ | 0·7 (l) | 0·6 (l) | 0·9 (l) | 1·1 (l) | 0·9 (l) | 0·9 (l) | 1·2 (vl) | 0·9 (l) |
| C3000/L | F+ | 0·4 (l) | 0·5 (l) | 0·6 (l) | 0·6 (s) | 0·5 (l) | 0·7 (l) | 1·8 (vl) | 0·6 (l) |
| 200Ps | Hfr | 0·5 (l) | 0·2 (l) | 0·2 (s) | 0·4 (s) | 0·2 (l) | 0·3 (l) | 0·6 (vl) | 0·5 (l) |
| 200Ps/L | Hfr | 0·3 (l) | 0·2 (l) | 0·2 (s) | 0·3 (s) | 0·2 (l) | 0·1 (l) | 0·2 (vl) | 0·4 (l) |
| C3000/RF | F+ | 0 | 0 | 0 | 0 | 0 | 0 | 2·0 (vl) | 0 |
| WF− | F− | 0 | 0 | 0 | 0 | 0 | 0 | 1·5 (vl) | 0 |
| WF+ | F+ | 0·6 (l) | 0·7 (l) | 0·9 (l) | 1·0 (l) | 1·1 (l) | 0·7 (l) | 1·8 (vl) | 0·2 (l) |
| 3300 | Hfr | 0·8 (l) | 0·9 (l) | 0·8 (s) | 0·9 (s) | 0·8 (l) | 0·8 (l) | 1·3 (vl) | 0·9 (l) |
| Q | F+ | 0·7 (l) | 0·6 (l) | 0·6 (s) | 0·6 (s) | 0·6 (l) | 0·6 (s) | 0·2 (vl) | 0·5 (l) |
| K10 | Hfr | 0·7 (s) | 0·8 (l) | 0·8 (s) | 0·8 (s) | 0·8 (l) | 0·8 (s) | 0·5 (vl) | 0·5 (l) |
| 40 | Hfr | 1·0 (s) | 1·0 (s) | 1·0 (s) | 1·0 (s) | 1·0 (s) | 1·0 (s) | 1·0 (vl) | 1·0 (l) |
| 40/L | Hfr | 0·3 (s) | 0·4 (s) | 0·8 (s) | 0·8 (s) | 0·6 (s) | 0·4 (s) | 2·9 (vl) | 0·6 (l) |

Table 2. *Sucrose-gradient sedimentation of sewage samples from Linlithgow and Stoke-on-Trent*

The sewage sample was shaken with chloroform and freed from large debris by centrifugation, and 1ml. was placed on top of a 24ml. sucrose gradient (20–5%, w/v) in TM1 buffer. Centrifugation at 96000$g_{max.}$ was maintained for 3hr. The gradients were fractionated by piercing the base of the tube and collecting 2ml. fractions. Then 1ml. samples of each fraction were plated with *E. coli* C3000/L by the double-layer agar technique. Duplicates were plated with an additional 0·1mg. of ribonuclease. Plates were incubated at 37° for 16hr.

| | | No. of plaques formed | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Fraction no. ... | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| Linlithgow | Ribonuclease-treated | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| | Untreated | 88 | 80 | 95 | 163 | 110 | 65 | 41 | 27 | 9 | 20 | 14 | 7 | 9 |
| Stoke-on-Trent | Ribonuclease-treated | 5 | 0 | 0 | 9 | 5 | 0 | 1 | 0 | 0 | 0 | 2 | 0 | 0 |
| | Untreated | 96 | 11 | 149 | 164 | 96 | 17 | 19 | 14 | 8 | 16 | 12 | 11 | 11 |

was determined in each part (Table 2). A plaque was picked at random from the plate of the third fraction of both gradients and purified by five cycles of plaque picking (Bradley, 1963). The complete inhibition of plaque formation by ribonuclease was checked after the final purification, and the morphology, homogeneity and purity of the RNA coliphage was checked by electron microscopy, as described by Bradley (1962). Both of the newly isolated RNA coliphages (ZS/3 from Stoke-on-Trent sewage, and ZL/3 from Linlithgow) were morphologically similar to ZIK/1, ZG, ZJ/1 and $\alpha$15 (Bradley, 1964).

*Preparation of* $^{32}$P-*labelled RNA coliphages.* The results obtained for one preparation of the RNA coliphage ZIK/1 are described in detail. The other RNA coliphages gave similar results.

*E. coli* C3000/L was inoculated from an exponentially growing broth culture into 30ml. of nutrient broth containing 0·95mc of [$^{32}$P]orthophosphate and incubated with shaking (70 oscillations/min.) at 37° for 3hr., during which time the extinction coefficient (measured in a Unicam SP.700 spectrophotometer; Unicam Instruments Ltd., Cambridge), read against a blank of nutrient broth, rose from zero to 0·2 (equivalent to a total of $2\times10^9$ bacteria). Then 0·33ml. of a sterile solution of $CaCl_2$ (0·1M) and 0·2ml. of a suspension of $10^{11}$ ZIK/1 were added to the culture and incubation was continued for 4·5hr. The extinction increased by 90min. to 0·68 (equivalent to a total of $18\times10^9$ bacteria) and then decreased, with the onset of lysis, to 0·05 (3hr. later). In pilot experiments this amount of ZIK/1 inoculum was found to be the minimum to ensure lysis and a resultant high titre of coliphage under the conditions used.

After bacterial debris had been removed by centrifugation in the MSE (Measuring and Scientific Equipment Ltd., Birmingham) Magnum refrigerated centrifuge at 5000*g* for 15min. (at 0°), the lysate was found to contain a total of

$8{\cdot}8 \times 10^{12}$ coliphages, as determined by the double-layer agar plating technique. Plaque formation was completely inhibited by the presence of 0·1 mg. of ribonuclease per plate.

The lysate was centrifuged at 78000*g* for 150 min. in the Spinco ultracentrifuge (rotor 30) to recover the coliphages. The translucent pellet was washed thrice by recentrifugation in 0·01 M-tris buffer, pH 7·2. Samples were removed from each of the four resulting supernatants to determine the content of $^{32}P$ and RNA coliphage (Table 3). After each centrifugation, the pellets were dispersed by passing 10–20 times through a wide-bore pipette. Finally the pellet was suspended in 5 ml. of TM1 buffer, and undispersed material was removed by centrifugation at 15000*g* for 15 min. (at 0°) in the MSE refrigerated centrifuge fitted with the superspeed attachment.

The supernatant ('coliphage suspension 1') contained $2{\cdot}8 \times 10^{12}$ coliphages (32% of the initial lysate) with a coliphages/(counts/sec.) ratio $3{\cdot}6 \times 10^{7}$ (Table 3). The coliphages/(counts/sec.) ratio was used as an empirical standard to determine the relative freedom of the coliphage preparation from soluble [$^{32}P$]phosphate-containing materials. The supernatants from the centrifugations at 78000*g* accounted for 5·6% of the initial viable coliphages (Table 3), so that, unless viability was diminished by the centrifugation, the residual 62% should have been in the 15000*g* pellet of unhomogenized material. To confirm this, the pellet was subjected to longer homogenization. A 20 ml. portion of TM1 buffer was added and the pellet homogenized by passing many times through a wide-bore pipette. The mixture was then stirred overnight at 0° with a magnetic stirrer and finally dialysed against five changes of TM1 buffer for 48 hr. at 0°. The resultant 'coliphage suspension 2' was assayed for content of radioactivity and coliphages (Table 3). It contained $4{\cdot}5 \times 10^{12}$ coliphages (51% of the initial lysate coliphages) and had a coliphages/(counts/sec.) ratio $2{\cdot}8 \times 10^{7}$.

Only the first coliphage suspension was used for subsequent purification and analyses.

Alternatively, the coliphages were suspended in 0·01 M-tris buffer, pH 7·2, containing $MgCl_2$ (0·1 mM) (TM2 buffer), and dialysed against the same solution for 24 hr. at 0° with five changes of liquid.

*Preparation of ribosomes.* Ribosomes were prepared from 1 l. of an overnight culture of unlabelled bacteria according to the method of Bishop, Roche & Nisman (1964). The final ribosome suspension, in TM1 buffer, had extinctions 7·8, 13·0 and 7·2 at 240, 260 and 280 m$\mu$ respectively, and was kept, without deterioration, in a sealed flask at 0° in the presence of chloroform for the duration of the experiments.

The procedure for labelling ribosomes with [$^{32}P$]phosphate was similar to that for the RNA coliphages. After inoculation of the nutrient-broth medium with bacteria and 1 mC of carrier-free [$^{32}P$]orthophosphate, incubation, with shaking, was maintained for 150 min. at 37°. The bacteria were then harvested by centrifugation at 5000*g* for 20 min. in the MSE refrigerated centrifuge. The cells were transferred to a mortar, cooled to −20° and 0·5 g. of alumina (A-305, bacteriological grade; Alcoa Chemicals, Pittsburgh, Pa., U.S.A.) was added. The alumina had previously been washed twice by centrifugation with TM1 buffer. While the cells were frozen, 0·5 ml. of TM1 buffer was added and the mixture ground with a cold pestle as it melted (2 min.). The contents were then refrozen and the process was repeated. Then 10 ml. of TM1 buffer was added, and large bacterial debris and alumina were removed from the mixture by centrifugation at 5000*g* for 20 min. Ribosomes were recovered from the supernatant by centrifugation at 78000*g* for 3 hr. They were resuspended in TM1 buffer and recentrifuged. This was repeated thrice. Finally, they were homogenized by pipetting in the same buffer and unhomogenized material was removed by centrifugation at 15000*g* for 15 min. in the MSE refrigerated centrifuge fitted with the superspeed attachment.

Alternatively, the ribosomes were homogenized, after the final washing, in TM2 buffer, and unhomogenized material was removed by centrifugation at 15000*g* for 15 min. The supernatant was then dialysed for 24 hr. at 0° against TM2 buffer with five changes of liquid.

*Sucrose-gradient sedimentation.* A 1 ml. portion of the preparation to be sedimented was placed on top of a 24 ml. linear gradient of sucrose (20–5% w/v), prepared in TM1 buffer or TM2 buffer (see below). Centrifugation and fractionation of the gradient were similar to that described above except that 0·8 ml. fractions (5–7 drops) were collected.

*Radioactivity.* The acid-insoluble radioactive material in fractions from the sucrose gradient was determined by mixing the samples (at 0°) with 2 ml. of 10% (w/v) tri-

Table 3. *Purification of ZIK/1 coliphage by differential centrifugation*

The preparation of the bacterial lysate and the methods of differential centrifugation are described in the Methods section. Coliphages were assayed by the double-layer agar plating technique. The radioactivity was measured after suitable dilution in distilled water (see the Methods section).

| | Coliphage count | $^{32}P$ (total counts/sec.) | Coliphages/(counts/sec.) ratio |
|---|---|---|---|
| Total lysate: | | | |
| 5000*g* supernatant | $8{\cdot}8 \times 10^{12}$ | $3{\cdot}30 \times 10^{7}$ | 2400 |
| 78000*g* supernatant 1 | $1{\cdot}3 \times 10^{11}$ | $3{\cdot}29 \times 10^{7}$ | 400 |
| 78000*g* supernatant 2 | $1{\cdot}1 \times 10^{11}$ | $8{\cdot}85 \times 10^{5}$ | $1{\cdot}3 \times 10^{5}$ |
| 78000*g* supernatant 3 | $1{\cdot}5 \times 10^{11}$ | $9{\cdot}32 \times 10^{3}$ | $1{\cdot}6 \times 10^{7}$ |
| 78000*g* supernatant 4 | $1{\cdot}0 \times 10^{11}$ | $3{\cdot}20 \times 10^{3}$ | $3{\cdot}1 \times 10^{7}$ |
| Coliphage suspension 1 | $2{\cdot}8 \times 10^{12}$ | $7{\cdot}5 \times 10^{4}$ | $3{\cdot}6 \times 10^{7}$ |
| Coliphage suspension 2* | $4{\cdot}5 \times 10^{12}$ | $1{\cdot}6 \times 10^{5}$ | $2{\cdot}8 \times 10^{7}$ |

* Corrected for decay of radioactivity during the course of the preparation.

chloroacetic acid, and collecting the precipitate on a 2cm. Oxoid membrane filter (Oxo Ltd.). The filters were washed thrice with 5ml. of 10% trichloroacetic acid, dried and counted for at least 500sec. with 5ml. of scintillator [primary phosphor 2,5-diphenyloxazole (0·5%), secondary phosphor 1,4-bis-(5-phenyloxazol-2-yl)benzene (0·03%), dissolved in toluene] in a Packard Tri-Carb liquid-scintillation spectrometer (model 3002) with the discriminator set at 50–1000 and gain at 0·9%. The counts were corrected for background activity. The residual radioactivity in the centrifuge tubes was measured by cutting the tubes into 1cm. squares, drying in a current of warm air (40°) and counting with 5ml. of scintillator.

Alternatively, the total content of radioactivity was determined by collecting the sucrose-gradient fractions in scintillation vials (Packard Instrument Co. Inc., La Grange, Ill., U.S.A.) and drying first in a current of warm air (40°) for 4hr. and then overnight in a desiccator over anhydrous $CaSO_4$; 5ml. of the scintillator was added and the samples were counted for at least 500sec. In the presence of sucrose it was necessary to correct for internal quenching, so that, after the initial counting, the scintillator was removed and the sample was washed twice with 5ml. of toluene, and a known quantity of [$^{32}$P]orthophosphate in 1ml. of water was added. The samples were dried, as above, recounted and a correction was made for internal quenching. The counts were also corrected for background activity.

Determination of the content of $^{32}$P in other material was made by placing a sample directly into a scintillation vial, adding a 1cm. square of filter paper, drying in a current of warm air (40°) and counting as described above.

*Alkaline hydrolysis.* After the counting, the membrane filter was washed with toluene to remove traces of scintillator. The toluene was then driven off in a current of warm air (40°) and the filter broken into about eight pieces in the base of a 7ml. MSE centrifugation tube. Then 50$\mu$l. of the unlabelled ribosome preparation and 0·5ml. of 1M-piperidine were added, the tubes sealed with aluminium foil and the contents hydrolysed at 60° for 36hr. (Crestfield & Allen, 1957).

Alternatively, fractions from the sucrose gradient were collected directly into 7ml. MSE centrifugation tubes and precipitated at 0° with 2ml. of 10% trichloroacetic acid. The acid-insoluble material was collected by centrifugation for 15min. at 15000$g$ (at 0°) in the MSE refrigerated centrifuge fitted with the super-speed attachment. The precipitate was washed thrice by suspending it in 5ml. of 10% trichloroacetic acid and centrifuging. The material was then hydrolysed as described above by piperidine after the addition of 50$\mu$l. of the suspension of unlabelled ribosomes.

By comparing the base ratios (see below) of samples precipitated before hydrolysis by 10% trichloroacetic acid in 7ml. MSE centrifugation tubes with the results obtained for samples precipitated and hydrolysed on membrane filters, it was found that the base ratios obtained by either method were, within experimental error, identical. Therefore hydrolysis of membrane filters did not affect the determination of the base ratio.

*Separation of nucleotides by paper chromatography.* The nucleotides liberated from RNA by alkaline hydrolysis were separated by two-dimensional chromatography on Whatman no. 1 paper at 18°. Isobutyric acid–water–aq. $NH_3$ (sp.gr. 0·88) (66:33:1, by vol.) containing 0·10% of EDTA (Magasanik, Vischer, Doniger, Elson & Chargaff, 1950) was used for the first development (18hr.) and the alcohol phase from 2-methylbutan-2-ol–aq. 90% (w/v) formic acid–water (3:1:3, by vol.) (Hanes & Isherwood, 1949) for the second development (48hr.).

Chromatograms were thoroughly dried in a current of warm air (40°). Nucleotides were located by visual examination under an ultraviolet lamp (Hanovia Ltd., Slough, Bucks.) Alkaline lysates were pale yellow owing to slight degradation of the membrane filters, but the degradation products (some of which fluoresced in ultraviolet light) moved with the solvent front on chromatography.

The ethanol–aq. 2% (w/v) boric acid–aq. $NH_3$ (sp.gr. 0·88) (6:3:1, by vol.) solvent system of Straus & Goldwasser (1961) was used to separate 2′,3′-ribonucleotides from 5′-ribonucleotides. Nucelotide spots from the two-dimensional chromatogram were cut out and sewn to 15in. strips of Whatman no. 1 paper, and the chromatograms developed in the descending fashion for 15hr. After drying, the location of the bases was determined from their ultraviolet absorption and the chromatograms were cut into $\frac{1}{2}$in. strips. Co-chromatography with standards [A-(mixed 2′,3′)-p, C-(mixed 2′,3′)-p, Cp, U-(mixed 2′,3′)-p, Up, G-(mixed 2′,3′)-p, Gp (Sigma Chemical Co.)] was used to determine $R_F$ values and separate the 2′,3′- from the 5′-ribonucleotides. At least 99·9% of each ribonucleotide present in the alkaline hydrolysate was the 2′,3′-ribonucleotide.

*Separation of ribonucleotides on Dowex 1 (formate form) columns.* Dowex 1 (X10; formate form; 200–400 mesh) (Dow Chemical Co., Midland, Mich., U.S.A.) columns were also used to separate ribonucleotides by a method similar to that of Hurlbert, Schmitz, Brumm & Potter (1954). Columns (0·6cm. × 11cm.) were washed successively with 250ml. of 3M-sodium formate, 100ml. of 1M-sodium formate–6N-formic acid, 100ml. of conc. (88%) formic acid and finally with distilled water until the eluent had pH4–5. Used columns were regenerated by washing through with 200ml. of 88% formic acid and then with distilled water.

The hydrolysate was evaporated to dryness in a current of warm air (40°), and applied to the column in distilled water. The column was washed with 20ml. of distilled water, and then ribonucleotides were eluted from the column with a formic acid gradient. The gradient was prepared by adding 30ml. of distilled water to the column and mixing chamber and then, while the chromatogram developed, 30ml. of 1N-formic acid, followed by 150ml. of 4N-formic acid. Fractions (3ml.) were collected at an elution rate of 0·3ml./min. The extinctions of the fractions at 260m$\mu$ (and 280m$\mu$ for cytidylic acid) were measured against blank solutions containing the same amount of formic acid. The fractions were then transferred to scintillation vials. A 1cm. square of Whatman no. 1 filter paper was added to each vial to prevent bumping, and the contents were evaporated to dryness on a thermostat hot-plate (Townson & Mercer Ltd., Croydon, Surrey). Then 5ml. of scintillator was added and the radioactivity measured for 500sec.

*Separation of ribonucleotides by paper electrophoresis.* The alkaline hydrolysate was evaporated to dryness in a current of warm air (40°) to remove the piperidine. It was then applied to Whatman 3MM filter-paper strips that had been soaked in potassium citrate buffer, pH3·5, and electrophoresis at a voltage gradient of 12–15v/cm. was carried out at 20–25° for 2·5–3·5hr. (U. E. Loëning, unpublished

work). The papers were dried in a current of warm air and ribonucleotides identified from their ultraviolet absorption.

*Determination of base ratios.* The $^{32}P$-labelled ribonucleotide spots on either the two-dimensional paper chromatograms or the paper-electrophoresis strips were cut from the paper, divided into 1 cm. squares, placed in the scintillation vials and counted in duplicate for 1000 sec. Neither the orientation of the papers nor the amount of paper in the vials affected the counting efficiency. The base ratio was determined from the sum of the counts/sec. of all four ribonucleotide spots and is expressed as a percentage of that sum. An estimate of the amount of unhydrolysed materials was obtained by similarly counting the origin spot of the chromatogram. The proportion of the total radioactivity of the original sample contained in the four $^{32}P$-labelled ribonucleotides was also determined after the counts on the chromatogram had been corrected for natural loss of radioactivity during the course of the experiment.

The radioactivity of the identified ribonucleotide fractions from the Dowex column was also used to determine base ratios.

*Preparation of anti-coliphage serum.* Anti-coliphage sera were prepared by standard techniques (Adams, 1959, p. 461), with rabbits as host. For example, a rabbit was inoculated at 10-day intervals with $10^{10}$, $10^{11}$ and $3 \times 10^{11}$ ZIK/1 coliphages that had been purified by differential centrifugation. At 14 days after the final inoculation, 10 ml. of blood was collected, allowed to clot (60 min.) and centrifuged at $2000g$ for 20 min. The serum was heated at 56° for 30 min. to remove complement, spun at $5000g$ for 15 min. and the supernatant stored at −20° until use.

*Neutralization of coliphage infectivity.* The serum was suitably diluted in 1·3% (w/v) nutrient broth and incubated with shaking at 37° with about $1 \times 10^8$ coliphages. Samples were removed at 5 min. intervals, diluted and assayed in duplicate to determine the residual number of infective coliphages.

## RESULTS

*Sucrose-gradient sedimentation of $^{32}P$-labelled RNA coliphages.* A 1 ml. portion of the ZIK/1 coliphage suspension 1 ($5{\cdot}6 \times 10^{11}$ coliphages; $1{\cdot}5 \times 10^4$ counts/sec.; see the Methods section) was fractionated in a sucrose gradient (20–5%, w/v) in TM 1 buffer, as described in the Methods section. A sample from each fraction was removed to determine the content of coliphages and the remainder used to determine the acid-insoluble radioactive material. There was a recovery of 98% of the coliphages and, including the residual radioactivity in the centrifuge tube (approx. 1·5%), a recovery of 97·5% of the total or acid-insoluble radioactivity. The coliphages were associated solely with the faster-moving of the two radioactive peaks (Fig. 1*a*). The coliphages/(counts/sec.) ratio was $8 \times 10^7$ for six of the main fractions of the first peak (all containing more than $2{\cdot}5 \times 10^{10}$ coliphages/fraction). Fractions with less than $2{\cdot}5 \times 10^{10}$ coliphages had lower ratios. The ratios for the fractions containing the second radioactive peak varied between $1 \times 10^5$ and $8 \times 10^5$. This suggested that the second peak was bacterial in origin and not associated specifically with the coliphages.

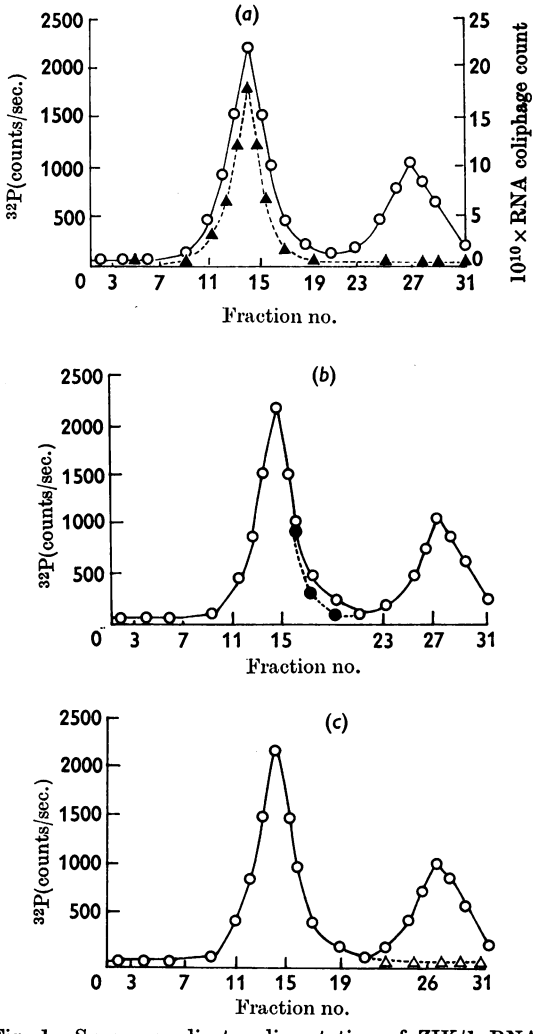


Fig. 1. Sucrose-gradient sedimentation of ZIK/1 RNA coliphage. A 1 ml. portion of ZIK/1 coliphage suspension 1 ($5{\cdot}6 \times 10^{11}$ coliphages; $1{\cdot}5 \times 10^4$ counts/sec.) was placed on top of a 24 ml. gradient of sucrose (20–5%, w/v) in TM 1 buffer and centrifuged at $90000g_{max.}$ for 3 hr. Fractionation of the gradient, determination of the acid-insoluble $^{32}P$-labelled material and RNA coliphage counts were performed as described in the Methods section. (*a*) Untreated ZIK/1 coliphage suspension in TM 1 buffer: ▲, RNA coliphage counts; ○, acid-insoluble $^{32}P$-labelled material. (*b*) ZIK/1 coliphage suspension in TM 1 buffer (○) and same pre-incubated at 37° for 60 min. with 50 $\mu$g. of deoxyribonuclease-free ribonuclease before centrifugation (●). (*c*) ZIK/1 coliphage suspension in TM 1 buffer (○) and same preincubated at 37° for 60 min. with 50 $\mu$g. of deoxyribonuclease before centrifugation (△).

To determine the amount of bacterial DNA and RNA in the preparation, 1ml. samples of the ZIK/1 coliphage suspension 1 were incubated at 37° for 60min. with 50$\mu$g. of deoxyribonuclease or ribonuclease (freed from deoxyribonuclease by heating at 90° for 5min.). The samples were then centrifuged and fractionated on sucrose gradients (20–5%, w/v) in TM1 buffer, as described above. Treatment with ribonuclease (Fig. 1*b*) decreased the recovery of acid-insoluble radioactive material to 91%. The coliphages/(counts/sec.) ratio of eight of the fractions (containing more than $1{\cdot}0\times10^{10}$ coliphages/fraction) was constant at $8\times10^{7}$. Treatment with ribonuclease did not affect the viability or recovery of the coliphages.

Treatment of the original coliphage preparation with deoxyribonuclease (Fig. 1*c*) decreased the recovery of acid-insoluble radioactive material to 65% but did not affect the recovery or viability of the coliphages. Only for those fractions with more than $2{\cdot}5\times10^{10}$ coliphages was the coliphages/(counts/sec.) ratio $8\times10^{7}$.

To determine the distribution of total radioactivity after deoxyribonuclease treatment, a 1ml. sample of radioactive coliphages was incubated similarly with 50$\mu$g. of deoxyribonuclease, placed on a sucrose gradient (20–5%, w/v) in TM1 buffer and centrifuged, as described above. It was found that 32% of the initial total radioactivity was present in the final three fractions of the gradient and 64% was distributed through the gradient, similarly to that recorded in Fig. 1(*c*) (broken line).

The proportion of the total radioactivity that was rendered acid-soluble by deoxyribonuclease treatment varied between 40 and 5% according to the preparation (even with preparations of the same coliphage). Correspondingly, the proportion of the total radioactivity present in the second peak of untreated samples varied with the preparation.

*Sucrose-gradient sedimentation of* $^{32}$P-*labelled ribosomes.* To estimate the contamination of the preparations by *E. coli* ribosomes, $^{32}$P-labelled ribosomes were prepared and isolated by methods as similar as possible to those used for the coliphage preparations. Centrifugation of the ribosomes with the coliphage also allowed an estimate to be made of the approximate sedimentation coefficients of the coliphages.

Preparations of $^{32}$P-labelled *E. coli* ribosomes in TM2 buffer or TM1 buffer were fractionated on a sucrose gradient in either TM2 buffer or TM1 buffer, as described above (Figs. 2*a* and 2*b*). The low concentration of $Mg^{2+}$ in TM2 buffer (Fig. 2*a*) resulted in the dissociation of high-molecular-weight into 50s and 30s ribosomes (Tissières, Watson, Schlessinger & Hollingworth, 1959). By comparison with the work of Schaechter (1963), the four peaks of $^{32}$P-labelled acid-insoluble material from the preparation in TM1 buffer (Fig. 2*b*) were identified as 140–120s, 100s, 70s and 50s ribosomes.

Preparations of the RNA coliphage ZIK/1 in TM1 buffer were incubated with 50$\mu$g. of deoxyribonuclease at 37° for 60min. and fractionated with and without additional ribosomes in TM1 buffer (Fig. 2*c*). Similarly, preparations in TM2 buffer were treated with deoxyribonuclease and fractionated with and without additional ribosomes in TM2 buffer (Fig. 2*d*).

These results (Fig. 2) indicate that the sedimentation coefficient of the ZIK/1 coliphage preparation was unaffected by variation in the concentration of magnesium chloride. By comparison with the ribosomes, it was observed that the ZIK/1 coliphage had a sedimentation coefficient of $80\pm5$s. Similar results and sedimentation coefficient were found for each of the other five RNA coliphages described in the present paper. Although there was a considerable overlap of the ribosomes (70s and 100s) and coliphage material in the presence of 0·01M-magnesium chloride (Fig. 2*c*, broken line) the overlap was much smaller for coliphage and dissociated ribosomes (50s and 30s) in the presence of 0·1mM-magnesium chloride (Fig. 2*d*, broken line). From Fig. 2(*a*) and the continuous line of Fig. 2(*d*) it was calculated that the ribosomal contamination in the 90–70s and 70–60s regions of the dialysed coliphage preparation would be no more than 1·5 and 25% respectively, i.e. no more than 4% for the whole 90–60s region. Although these values are approximate and would vary from preparation to preparation, it was apparent that substantial ribosomal contamination occurred in the 70–60s coliphage region. This would cause inaccuracies in the base-ratio determinations of the whole coliphage region (90–60s).

To decrease the amount of contamination, 1ml. of dialysed ZIK/1 coliphage preparation in TM2 buffer (1340 counts/sec.) was incubated for 60min. at 37° with 50$\mu$g. each of ribonuclease and deoxyribonuclease. After incubation, the preparation was centrifuged for 3hr. in a sucrose gradient and fractionated. The total amounts of acid-insoluble radioactive material in the 70–60s and 60–25s regions were respectively 6 and 4% of that in the 90–70s region. By comparison with Fig. 2(*a*) it was calculated that, after ribonuclease treatment, no more than 0·5 and 8% respectively of the total acid-insoluble radioactive material of the 90–70s and 70–60s fractions could be due to ribosomal contamination, i.e. no more than 1% for the whole region. This amount of contamination would not substantially affect determinations of the base ratios (see the next section).

*Determination of base ratios by paper chromato-*

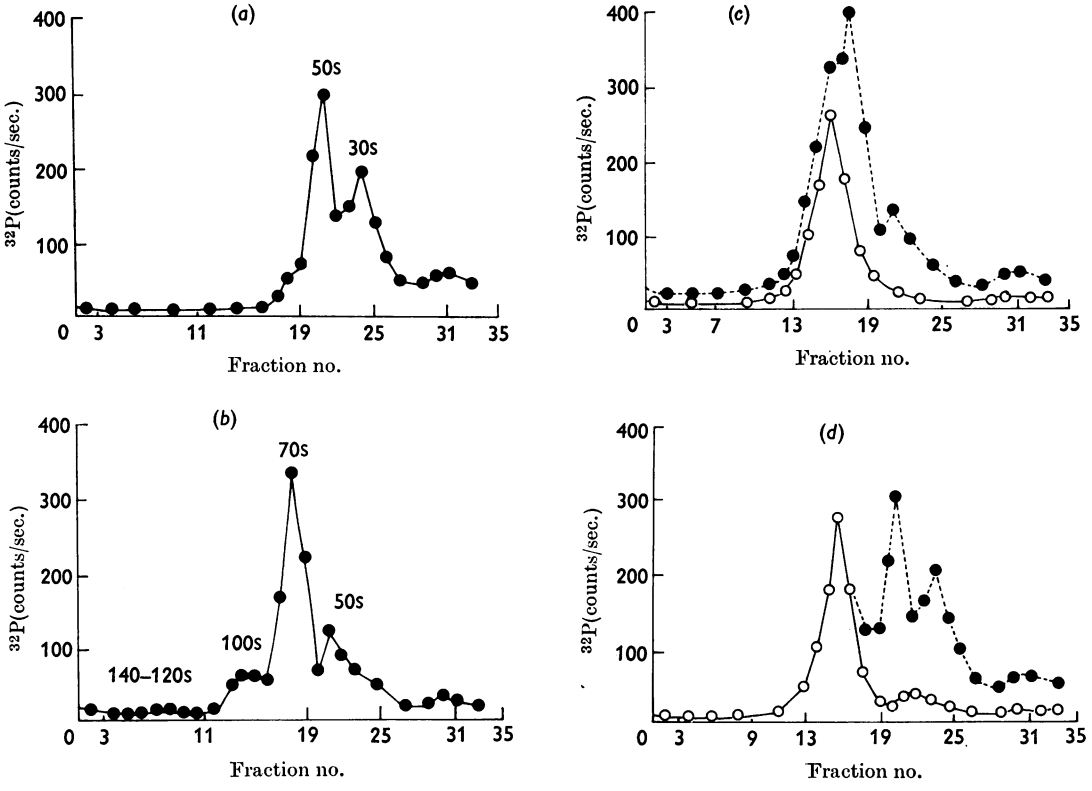


Fig. 2. Sucrose-gradient sedimentation of C3000/L *E. coli* ribosomes and ZIK/1 RNA coliphage. A 1 ml. portion of ribosome preparation or 1 ml. of deoxyribonuclease-treated ZIK/1 RNA coliphage or 1 ml. of each were centrifuged and fractionated, and the acid-insoluble radioactivity was determined as in Fig. 1. Sucrose gradients were in either TM1 buffer or TM2 buffer, as appropriate. (*a*) A 1 ml. portion of dialysed $^{32}$P-labelled ribosomes (1750 counts/sec.) in TM2 buffer. (*b*) A 1 ml. portion of undialysed $^{32}$P-labelled ribosomes (2060 counts/sec.) in TM1 buffer. (*c*) A 1 ml. portion of undialysed $^{32}$P-labelled ZIK/1 RNA coliphage (1250 counts/sec.) in TM1 buffer (○, continuous line), and mixed with 1 ml. of undialysed $^{32}$P-labelled ribosomes (2060 counts/sec.) in TM1 buffer (●, broken line). (*d*) A 1 ml. portion of dialysed $^{32}$P-labelled ZIK/1 RNA coliphage (1340 counts/sec.) in TM2 buffer (○, continuous line), and mixed with 1 ml. of dialysed $^{32}$P-labelled ribosomes (1750 counts/sec.) in TM1 buffer (●, broken line).

*graphy*. For each of the six RNA coliphages, base ratios were determined on coliphage fractions purified by sucrose-gradient centrifugation and precipitated on membrane filters with 10% trichloroacetic acid. To minimize the amount of DNA and ribosomal contamination, the coliphage preparations, before centrifugation, were incubated for 60 min. at 37° with deoxyribonuclease (50 $\mu$g./ml.) and ribonuclease (50 $\mu$g./ml.) and then dialysed against TM2 buffer for 24 hr. at 0° with five changes of liquid. The sucrose gradients were also prepared in TM2 buffer.

To determine the importance, for the subsequent determinations of base ratios, of purifying the coliphage preparation by treating with deoxyribonuclease, ribonuclease and dialysis, an undialysed untreated ZIK/1 coliphage preparation (6944·4 counts/sec.) was centrifuged in a sucrose gradient (20–5%, w/v) in TM1 buffer, fractionated and the RNA base ratio determined on the acid-insoluble material of each fraction. After precipitation with 10% (w/v) trichloroacetic acid at 0°, the acid-insoluble material was collected on Oxoid membrane filters and, after alkaline hydrolysis, the hydrolysate chromatographed by two-dimensional paper chromatography. The results for two-thirds of the gradient are given in Table 4.

The amount of radioactivity present in the ribonucleotide spots of the two-dimensional paper chromatogram was 94–97% of the original for the coliphage fractions of the sucrose gradient (fractions 9–18). The recovery then decreased in the

Table 4. *Determination of base ratios of a crude undialysed ZIK/1 coliphage preparation after sucrose-gradient centrifugation*

The ZIK/1 coliphage suspension, in TM1 buffer (6944·4 counts/sec.), was centrifuged in a gradient of sucrose, fractionated (column 1) and precipitated on membrane filters. The determination of radioactivity (column 2) and alkaline hydrolysis of the fractions are described in the text and Methods section. The determination of base ratios was made after two-dimensional paper chromatography of the alkaline hydrolysate to resolve the ribonucleotides. Ribonucleotide spots were cut from the paper and counted as described in the Methods section. Base ratios are expressed as percentages of the total radioactivity in the ribonucleotide spots. The recoveries of $^{32}P$ in the ribonucleotides (column 4) are expressed as percentages of the original acid-insoluble radioactivity. The amounts of added radioactivity that remained at the origin of the chromatogram are also expressed as percentages of the added radioactivity (last column).

| Fraction no. | Acid-insoluble radioactivity (counts/sec.) | RNA base ratio (%) | | | | Percentage recovery of radioactivity | |
|---|---|---|---|---|---|---|---|
| | | A | C | G | U | In ribonucleotide spots | At origin |
| 9 | 53·5 | 23·9 | 23·9 | 26·1 | 26·1 | 95 | 1 |
| 10 | 131·6 | 23·1 | 24·6 | 23·5 | 28·8 | 95 | 1 |
| 11 | 252·8 | 22·8 | 24·9 | 23·9 | 28·4 | 96 | 1 |
| 12 | 449·3 | 24·3 | 23·6 | 24·2 | 27·9 | 96 | 1 |
| 13 | 760·0 | 23·7 | 24·2 | 24·0 | 28·1 | 97 | 0·5 |
| 14 | 1143·7 | 23·5 | 24·3 | 23·9 | 28·3 | 97 | 0·5 |
| 15 | 735·2 | 23·6 | 23·8 | 23·7 | 27·9 | 97 | 0·5 |
| 16 | 471·8 | 23·9 | 23·7 | 24·4 | 28·0 | 95 | 1 |
| 17 | 203·9 | 24·4 | 23·6 | 25·5 | 26·5 | 95 | 2 |
| 18 | 135·6 | 24·7 | 23·3 | 26·1 | 25·9 | 94 | 3 |
| 19 | 92·3 | 25·2 | 22·0 | 29·7 | 23·1 | 89 | 7 |
| 20 | 71·4 | 25·1 | 22·1 | 29·9 | 22·9 | 84 | 11 |
| 21 | 48·1 | 25·5 | 21·6 | 29·3 | 23·6 | 78 | 18 |
| 22 | 53·6 | 25·3 | 22·1 | 29·4 | 23·2 | 59 | 34 |
| 23 | 95·9 | 24·9 | 22·6 | 27·5 | 25·0 | 25 | 70 |
| 24 | 145·3 | 24·6 | 23·2 | 27·0 | 25·2 | 10 | 84 |
| 25 | 223·1 | 24·1 | 24·1 | 26·0 | 25·8 | 5 | 89 |
| 26 | 310·6 | 21·9 | 23·9 | 28·3 | 25·9 | 3 | 92 |
| 27 | 401·9 | 23·9 | 24·2 | 26·7 | 25·2 | 2 | 96 |
| 28 | 325·5 | 23·9 | 22·9 | 27·2 | 26·0 | 4 | 92 |
| 29 | 261·2 | 24·2 | 23·1 | 26·9 | 25·8 | 5 | 91 |
| 30 | 193·3 | 23·7 | 22·9 | 27·4 | 26·0 | 5 | 89 |
| 31 | 104·8 | 24·5 | 23·0 | 28·2 | 24·3 | 15 | 81 |

succeeding fractions and was minimal (5–2%) in the deoxyribonuclease-sensitive fractions forming the second radioactive peak (fractions 25–30). Correspondingly, the amount of the original radioactivity remaining on the origin spot of the chromatogram, after paper chromatography, was minimal (0·5–3%) in the coliphage region but maximum in the deoxyribonuclease-sensitive peak. In view of the observation (see below) that the amount of radioactivity remaining at the chromatogram origin was decreased after deoxyribonuclease treatment, it was apparent that this material was in fact DNA. The average base ratios (and S.E.M.) for six of the main coliphage fractions (10–15 inclusive) were $23·5 \pm 0·5$, $24·2 \pm 0·4$, $23·9 \pm 0·2$ and $28·2 \pm 0·4$ for A, C, G and U respectively. Fractions before and after these fractions had a higher content of G; the base ratios of the fractions after the main coliphage fractions (19–22) were similar to the values given to ribosomes by Spahr & Tissières (1959) (i.e. for 70S ribosomes $25·0 \pm 0·5$, $22·1 \pm 0·2$, $31·5 \pm 0·6$ and $21·4 \pm 0·6$ for A, C, G and U respectively). The higher values for U of these fractions (19–22) by comparison with authentic ribosomes was presumably a reflection of the fact that the fractions contained a mixture of ribosomes and coliphage. The base ratios of the fractions 23–31 varied considerably. All, however, contained more G than any other ribonucleotide.

A similar experiment was performed in which the crude coliphage preparation in TM2 buffer (6357·5 counts/sec.) was treated with deoxyribonuclease and ribonuclease and then dialysed before centrifugation in a sucrose gradient (20–5%, w/v) in TM2 buffer. The base ratios were determined similarly to the previous experiment (Table 5). Fractions 22–30 contained less than 9 counts/sec., and consequently analyses of base ratios were

Table 5. *Determination of base ratios of a ZIK/1 coliphage preparation treated with deoxyribonuclease and dialysed against* 0·01 M-*tris buffer*, pH 7·2, *containing magnesium chloride* (0·1 mM)

The methods of determining base ratios are the same as in Table 4. The ZIK/1 coliphage preparation in TM2 buffer (6357·5 counts/sec.) was treated with 50 $\mu$g. of deoxyribonuclease/ml. and 50 $\mu$g. of ribonuclease/ml. at 37° for 60 min. and then dialysed for 24 hr. against TM2 buffer with five changes of liquid. The preparation was then placed on a sucrose gradient (20–5%, w/v) in TM2 buffer and fractionated as described in the Methods section.

| Fraction | Acid-insoluble radioactivity (counts/sec.) | RNA base ratio (%) | | | | Percentage recovery of radioactivity | |
|---|---|---|---|---|---|---|---|
| | | A | C | G | U | In ribonucleotide spots | At origin |
| 8 | 43·7 | 23·7 | 24·1 | 23·7 | 28·5 | 96 | 1 |
| 9 | 147·6 | 23·8 | 24·3 | 23·8 | 28·1 | 96 | 1 |
| 10 | 211·6 | 23·7 | 24·3 | 23·8 | 28·2 | 96 | 0·5 |
| 11 | 383·0 | 23·6 | 24·0 | 23·9 | 28·5 | 96 | 1 |
| 12 | 737·6 | 23·1 | 24·3 | 24·0 | 28·6 | 97 | 0·5 |
| 13 | 1234·8 | 23·6 | 24·1 | 23·9 | 28·4 | 97 | 0·5 |
| 14 | 755·9 | 23·7 | 24·0 | 24·0 | 28·3 | 96 | 0·5 |
| 15 | 430·9 | 24·0 | 23·9 | 23·7 | 28·2 | 97 | 1 |
| 16 | 141·6 | 23·9 | 23·9 | 24·0 | 28·2 | 97 | 1 |
| 17 | 62·1 | 23·5 | 23·9 | 24·1 | 28·5 | 98 | 1 |
| 18 | 41·8 | 23·9 | 23·8 | 24·1 | 28·2 | 97 | 1 |
| 19 | 23·9 | 24·0 | 23·6 | 24·5 | 27·9 | 97 | 2 |
| 20 | 16·2 | 24·1 | 23·7 | 25·6 | 26·6 | 96 | 1 |
| 21 | 9·1 | 24·0 | 23·1 | 26·3 | 26·6 | 97 | 0·5 |

not attempted on these fractions. The recovery of radioactivity in the ribonucleotide spots for fractions 8–21 inclusive was 96–98%, whereas 0·5–2% remained at the origin. The RNA base ratios (and S.E.M.) of the eight main coliphage fractions (9–16 inclusive) were $23·7 \pm 0·3$, $24·1 \pm 0·2$, $23·9 \pm 0·1$ and $28·3 \pm 0·2$ for A, C, G and U respectively.

It was apparent from these two sets of experiments (Tables 4 and 5) that pretreatment with deoxyribonuclease and ribonuclease resulted in coliphage preparations that, after fractionation in a gradient of sucrose, gave more uniform base ratios for the main coliphage fractions and also contained less bacterial DNA or RNA.

The base ratios obtained by these procedures for all six RNA coliphages are given in Table 6. The base ratios obtained for 70S ribosomes isolated from a sucrose gradient of undialysed ribosomes (see Fig. 2*b*) are also given in Table 6. Different preparations of the same coliphage gave, within experimental error, the same base ratios.

There was no substantial difference in the base ratios obtained for the coliphages when [$^{32}$P]orthophosphate was added only 30 min. before the addition of coliphages to an exponentially growing *E. coli* culture. Quantitatively, though, it was found that the coliphages/(counts/sec.) ratio was higher for the purified coliphage fractions after sucrose-gradient centrifugation when [$^{32}$P]orthophosphate was added 30 min. before the addition of coliphages. Similarly, when, during the preparation of $^{32}$P-labelled ribosomes, the [$^{32}$P]orthophosphate was added to the exponentially growing bacteria 90 min. before they were harvested, the base ratios were not substantially different from those prepared after a longer labelling period.

*Determination of base ratios by column chromatography and paper electrophoresis.* The RNA base ratios of material in the alkaline hydrolysate of coliphage fractions were also determined by column chromatography and paper electrophoresis. The values found were essentially the same as those determined by paper chromatography (Table 6).

(*a*) Column chromatography. Dowex 1 (formate form) columns were prepared and used as described in the Methods section. A ZS/3 coliphage sample, purified by sucrose-gradient centrifugation after deoxyribonuclease, ribonuclease and dialysis treatment, was precipitated with 10% trichloroacetic acid, filtered and counted on a membrane filter (768·2 counts/sec.). Unlabelled ribosomes were added to the coliphage sample and the mixture was hydrolysed by piperidine and applied to the Dowex column as described above. After washing with 20 ml. of water, which removed the yellowish degradation products of the membrane filter, 40 fractions (3 ml.) were collected on elution of the column by a formic acid gradient (see the Methods section). The ribonucleotides, identified by their extinctions at 240, 260 and 280 m$\mu$, were eluted in the following order: C (fractions 6–10), A (fractions 12–17), G (fractions 23–29) and U (fractions 31–38). The total amount of radioactivity in these fractions

Table 6. *Base ratios of 70s ribosomes and six RNA coliphages*

The methods of determining base ratios on coliphage or ribosome samples, purified by sucrose-gradient centrifugation, are given in the text. Samples were acid-precipitated on membrane filters and hydrolysed with piperidine, and the ribonucleotides separated by two-dimensional paper chromatography (see the text). Base ratios are calculated as percentages of the total radioactivity contained in the ribonucleotides. The S.E.M. is also given. The numbers of separate sample preparations and the number of analyses used to obtain the base ratios are given. The host bacterium was preincubated with [$^{32}$P]orthophosphate for 3 hr. before addition of the coliphage.

| Sample | | RNA base ratio (%) A | C | G | U | Purines / Pyrimidines ratio | A+C / G+U ratio | A+U / G+C ratio |
|---|---|---|---|---|---|---|---|---|
| ZIK/1 | 3 preparations; 30 analyses | 23·6±0·5 | 24·2±0·3 | 23·9±0·3 | 28·3±0·3 | 0·90 | 0·92 | 1·08 |
| ZJ/1 | 2 preparations; 24 analyses | 24·3±0·5 | 23·7±0·3 | 23·8±0·2 | 28·2±0·3 | 0·93 | 0·92 | 1·11 |
| ZG | 2 preparations; 30 analyses | 25·1±0·6 | 24·0±0·4 | 27·2±0·3 | 23·7±0·3 | 1·10 | 0·96 | 0·95 |
| ZS/3 | 2 preparations; 20 analyses | 24·8±0·6 | 23·8±0·4 | 28·0±0·4 | 23·4±0·4 | 1·12 | 0·95 | 0·93 |
| ZL/3 | 2 preparations; 20 analyses | 24·8±0·7 | 24·9±0·5 | 26·9±0·3 | 23·4±0·4 | 1·07 | 0·99 | 0·93 |
| α15 | 2 preparations; 20 analyses | 24·7±0·6 | 25·0±0·3 | 26·3±0·3 | 24·0±0·4 | 1·04 | 0·99 | 0·95 |
| 70s Ribosomes | 2 preparations; 10 analyses | 25·5±0·5 | 21·9±0·4 | 30·8±0·3 | 21·8±0·3 | 1·29 | 0·90 | 0·90 |

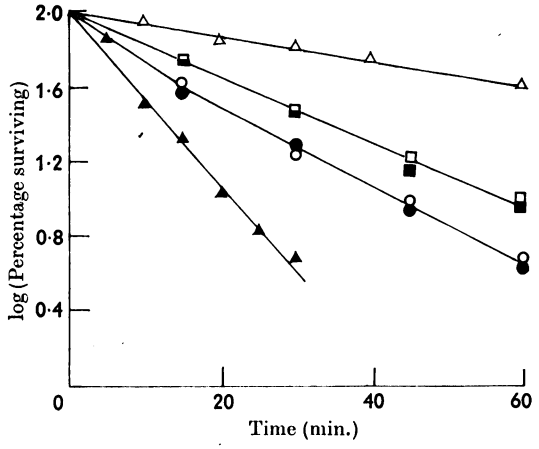


Fig. 3. Neutralization of RNA coliphages by anti-(ZIK/1 coliphage) serum. Details of the preparation of the anti-(ZIK/1 coliphage) serum and determination of the number of coliphages are given in the Methods section. The serum, suitably diluted in 1·3% (w/v) nutrient broth (5 ml.), was incubated at 37° with $1 \times 10^8$ RNA coliphages. Samples were removed at 5 min. intervals and the residual coliphages determined, after dilution, by the double-layer agar plating technique. The number of survivors are plotted as the logarithm of the percentage remaining. Serum dilution 1:50000: ▲, ZIK/1 RNA coliphage; △, ZJ/1 RNA coliphage. Serum dilution 1:500: □, α15 RNA coliphage; ■, ZL/3 RNA coliphage; ○, ZG RNA coliphage; ●, ZS/3 RNA coliphage.

were respectively 170·0, 181·4, 199·3 and 169·2 counts/sec., equivalent to base ratios 23·6, 25·2, 27·7 and 23·5% for C, A, G and U ribonucleotides respectively. Approx. 6% of the added radioactivity did not occur in the ribonucleotide fractions. Duplicate experiments gave similar results.

(*b*) Paper electrophoresis. A ZS/3 coliphage sample was similarly prepared and hydrolysed, and the hydrolysate dried and applied to Whatman 3MM filter-paper strips soaked in potassium citrate buffer, pH 3·5, and electrophoresis carried out as described (see the Methods section). The base ratios expressed as percentages of the total radioactivity in the ribonucleotides were 24·8, 23·9, 27·7 and 23·5% for A, C, G and U respectively. Separation of A from C was not always satisfactory, but on the occasions when the separation was unsatisfactory the ratios were 48·8, 27·8 and 23·4% for A + C, G and U respectively. Approx. 5% of the initial radioactivity was not found in the ribonucleotide spots.

*Antigenic properties.* Fig. 3 shows the results of incubating the six RNA coliphages with anti-(ZIK/1 coliphage) serum at serum dilutions 1:50000 (for ZIK/1 and ZJ/1) and 1:500 (for ZS/3, ZL/3, ZG and α15). For the ZIK/1 anti-

coliphage serum, the velocity constants of phage neutralization, calculated by the method of Adams (1959, p. 463) from the percentage inactivation at various times and serum dilutions and expressed as the average of the values obtained, were 5000 for ZIK/1, 770 for ZJ/1, 27 for ZS/3, 25 for ZG, 22 for ZL/3 and 19 for $\alpha 15$.

Similarly, with the anti-(ZG coliphage) serum the velocity constants of coliphage neutralization were 2200 for ZG, 1450 for ZS/3, 900 for ZL/3, 600 for $\alpha 15$, 15 for ZIK/1 and 12 for ZJ/1. With the anti-($\alpha 15$ coliphage) serum the velocity constants of coliphage neutralization were 1500 for $\alpha 15$, 1300 for ZL/3, 950 for ZS/3, 650 for ZG, 10 for ZIK/1 and 10 for ZJ/1. For all three antisera the rate of neutralization was greater for the homologous coliphage and antiserum than with heterologous coliphages. In view of this and the fact that the coliphages were isolated from widely separated sources and purified by several cycles of plaque picking, it is apparent that none of the six coliphages are identical. Further, the differences in neutralization characteristics indicate that the coliphages ZIK/1 and ZJ/1 are similar to each other but are serologically dissimilar to the other four coliphages. The coliphages ZG, ZS/3, ZL/3 and $\alpha 15$ are apparently serologically related, but may fall into two subgroups (i.e. ZG+ZS/3 and $\alpha 15$+ZL/3). This latter conclusion is, however, only tentative, and must await more detailed peptide analysis of the antigenic proteins of the respective coliphages.

## DISCUSSION

The validity of determining base ratios by labelling the RNA with [$^{32}$P]phosphate and separating the ribonucleotides after alkaline hydrolysis depends on the assumption that [$^{32}$P]phosphate in the medium becomes randomly incorporated into the RNA in the cell. This assumption can be verified by comparing base ratios determined by $^{32}$P-labelling with those obtained by other methods. It has been demonstrated that the determination of the base ratios of 70s ribosomes labelled with $^{32}$P (under similar conditions to those employed for coliphage preparation) are in reasonable agreement with those obtained by extinction measurements of ribonucleotides separated by column chromatography of an alkaline hydrolysate of *E. coli* 70s ribosomes (Spahr & Tissières, 1959). The demonstration that the ribonucleotides of the alkaline hydrolysate are the 2′,3′-nucleotides and not the 5′-nucleotides confirms the validity of this method of determining base ratios and also indicates that there are no bound 5′-nucleotides in the preparations analysed (Straus & Goldwasser, 1961; Cummins & Plaut, 1964).

From the determinations of the base ratios, and the observation that the percentages of A and U or G and C are not equivalent for any of the RNA coliphages, it is concluded that the coliphages described in the present paper contain single-stranded RNA.

The RNA coliphage (f2) isolated by Loeb & Zinder (1961) had base ratios (calculated from their results) 22·2, 26·8, 25·9 and 25·1% for A, C, G and U respectively, with ratios 0·93 for (A+G)/(C+U), 0·96 for (A+C)/(G+U), and 0·90 for (A+U)/(G+C). The RNA coliphage (R17) examined by Paranchych & Graham (1962) had base ratios (calculated from their results) 22·6, 24·8, 27·1 and 25·5% for A, C, G and U respectively, with ratios 0·99 for (A+G)/(C+U), 0·90 for (A+C)/(G+U), and 0·93 for (A+U)/(G+C). The RNA coliphage (MS2) examined by Strauss & Sinsheimer (1963) had, after alkaline hydrolysis, base ratios 22·4, 24·5, 27·5 and 25·6% for A, C, G and U respectively, with ratios 1·0 for (A+G)/(C+U), 0·88 for (A+C)/(G+U), and 0·92 for (A+U)/(G+C). Finally, the RNA coliphage (fr) examined by Marvin & Hoffmann-Berling (1963) had base ratios 24·9, 24·3, 27·1 and 23·7% for A, C, G and U respectively, with ratios 1·06 for (A+G)/(C+U), 0·97 for (A+C)/(G+U), and 0·92 for (A+U)/(G+U).

Of these four RNA coliphages only the last shows any similarity to the six investigated in the present paper. The similarity of the base ratios of RNA coliphage fr to those of ZL/3 and $\alpha 15$ suggests that fr could be related to these two coliphages. The degree of relativity cannot, however, be determined by determining base ratios alone. It is possible that two similar RNA base ratios could give rise to two dissimilar proteins.

One criteria of relativity is the antigenic properties of a coliphage. These antigenic properties are determined by the coat proteins (Adams, 1959, p. 97). Of the six coliphages examined, two (ZIK/1 and ZJ/1) are antigenically dissimilar to the other four, but to some extent similar to each other. The other four (ZS/3, ZL/3, $\alpha 15$ and ZG) are serologically related to each other. Further, the base ratios of ZIK/1 and ZJ/1 are similar to each other, and the base ratios of ZS/3, ZG, $\alpha 15$ and ZL/3 are also similar to each other. Whether these similarities represent similar coat proteins in the two or three groups of coliphages will have to await detailed peptide analysis.

Finally, the RNA coliphages analysed in the present work are morphologically similar and appear under the electron microscope to have a regular hexagonal outline and a diameter of 225 Å. (A more detailed analysis of the physical structure of RNA coliphages in electron micrographs is given by Bradley, 1964.)

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