

ON THE FUNCTION AND FATE OF PHAGE PROGENY RNA IN INFECTED BACTERIA

M. L. FENWICK

*Sir William Dunn School of Pathology,
University of Oxford, England*

SUMMARY

Spheroplasts of *Escherichia coli* infected with RNA phage R 17 were treated with actinomycin D to inhibit the synthesis of host RNA and protein. Ribosomes occurred predominantly as monomers, dimers and trimers which were active in viral protein synthesis. Partly finished strands of viral RNA in lysates sedimented at a rate characteristic of groups of 6-12 ribosomes and their buoyant density suggested that they were in fact attached to ribosomes. Newly completed strands were associated with single (70-s) ribosomes, forming a 75-s complex, and with ribosome dimers and trimers. Some were probably loosely bound to 30-s ribosomal subunits.

Newly made RNA began to accumulate in mature virions after a lag of about 5 min and rose to 16% of the total labelled viral RNA by the end of the growth cycle, either with continuous labelling or after a 5-min pulse followed by a non-radioactive chase. It is suggested that most, if not all, new viral RNA forms a rather stable association with ribosomes and that a small proportion of this RNA may subsequently pass into progeny virus.

INTRODUCTION

The nucleic acid of an RNA virus must be able to perform three functions: (1) direction of the synthesis of virus-specific proteins, (2) direction of the synthesis of copies of itself and (3) transport of the information for these processes to a new host cell in the form of mature virions. The original infecting strand of RNA must accomplish in turn at least the first and second of these, but there is evidence (Davis & Sinsheimer, 1963) that in the case of phage MS 2 it is not subsequently re-incorporated into a mature virus particle amongst its progeny. We have examined the newly made progeny RNA molecules to try to determine what are the relative likelihoods of their performing functions (1) and (3) and whether the same RNA strand can do both.

Infection of *E. coli* with the RNA phage R 17 causes a loss of the larger polysomes and accumulation of single ribosomes (Hotham-Iglewski, Phillips & Franklin, 1968). The use of phage-infected spheroplasts treated with actinomycin D makes possible the study of virus growth uncomplicated by a background of host RNA and protein synthesis (Kelly, Gould & Sinsheimer, 1965; Vinuela, Algranati & Ochoa, 1967). We have used sucrose gradient centrifugation to show that in such a system single ribosomes and small polysomes make viral protein, and to observe the appearance in them, and in mature virions, of newly made phage RNA.

MATERIALS AND METHODS

Infection and lysis

Escherichia coli K12, strain Hfr₁, was grown in tris-buffered medium containing casamino acids and glucose and infected with the RNA phage R17 (see Erikson, Fenwick & Franklin, 1964). Cultures of bacteria were grown to about 4×10^9 cells in 10 ml, chilled in ice and infected with 20 plaque-forming units of phage per cell. After 10 min the culture was warmed and incubated for a further 10 min at 37 °C, chilled again, centrifuged and converted to spheroplasts with lysozyme as described before (Fenwick, 1968*a*), but without chloramphenicol (CAP). The spheroplasts were diluted into 9 volumes (4.5 ml) of growth medium at 37 °C containing 0.01 M magnesium acetate and 10% (w/v) sucrose, and actinomycin D (0.5 µg/ml, kindly supplied by Merck, Sharpe and Dohme, Inc. New Jersey). This time was designated 10 min after infection. Incubation with slow shaking was continued and terminated by pouring the culture on to an equal volume of crushed frozen medium containing 0.01 M Mg²⁺, 10% sucrose and CAP, 200 µg/ml.

The spheroplasts were centrifuged in the cold and resuspended in 0.225 ml of 0.01 M magnesium acetate with 0.01 M or 0.03 M sodium phosphate, pH 7.2. They were lysed by adding 0.025 ml of 5% (w/v) Brij 58 (Honeywell and Stein Ltd., Carshalton, Surrey) and the whole lysate applied to a sucrose gradient. The phosphate buffer, pH 7.2, was prepared by mixing 0.2 M NaH₂PO₄ and 0.2 M Na₂HPO₄. It was used in preference to tris buffer since formaldehyde (see below) reacts with tris.

Sucrose gradients

The lysate was applied to a sucrose gradient containing the same concentration of magnesium and phosphate and centrifuged at 50 000 rev/min in a Spinco SW 50.1 rotor at 10 °C. Exponential gradients of 30–10% (w/v) sucrose (4.8 ml) were made and monitored for optical density at 254 nm as described before (Fenwick, 1968*b*).

Caesium chloride gradients

Fractions collected from a sucrose gradient were fixed by adding neutralized 36% formaldehyde to give a concentration of 4% for 4–5 h at 4 °C (Spirin, Belitsina & Lerman, 1965). The fixed ribosomes were made up to 1 ml with 0.01 M Mg²⁺, 0.01 M phosphate, enough formaldehyde to make a final concentration of 2% in the gradient, and Brij 58 to 0.1%. CsCl was added (2.0 ml of a saturated solution at 20 °C) and the mixture was overlaid with 2.0 ml of heptane to prevent collapse of the tube during centrifugation at 10 °C in a Spinco rotor SW 50.1 at 35 000 rev/min for 15 h. Saturated CsCl solution was used to displace the gradient for monitoring and fraction collecting. The densities of the fractions were determined by weighing in a 0.05-ml pipette at 20 °C.

Radioactivity and counting

Uridine-5-³H (5 Ci/mM) and ³²P-phosphate (60 Ci/mg P) were obtained from the Radiochemical Centre, Amersham. For pulse-labelling 20 µCi [³H]uridine/ml was used; for 10 min or longer periods, 2–4 µCi/ml with 2.5 µg unlabelled uridine/ml. Ribosomal RNA was labelled by growing bacteria for 1 h with 1–2 µCi ³²P/ml.

For measurement of radioactivity, fractions from a gradient were collected on Whatman GF/A glass fibre filters (2 cm diameter) suspended on pins set in the perspex turntable of a fraction collector. The turntable, with the filters still wet, was then placed on a horizontal axle and revolved at 3 rev/min so that the lower filters dipped beneath the surface of a bath of 10% (w/v) trichloroacetic acid (TCA) at 20 °C. After 5 min the turntable was moved to a bath of 5% TCA for a further 5 min, and finally to 95% ethanol for 5 min. The filters were then dried at 37 °C, placed in glass specimen tubes and covered with 2.5 ml of scintillation fluid. The position of the filters, if immersed, was immaterial. The sealed tubes were placed inside standard scintillation vials for counting (with about 20% efficiency for ³H).

RESULTS

Distribution of polysomes

The distribution in sucrose gradients of ribosomes from uninfected bacteria is shown in Fig. 1. In the presence of 0.01 M magnesium acetate, 0.01 M sodium phosphate (Fig. 1A), there is a large peak of 70-s ribosomes and the polysomes show a maximum of optical density in the vicinity of groups of 8 ribosomes. Lysates prepared and centrifuged in 0.01 M Mg^{2+} , 0.03 M phosphate (Fig. 1B), display a marked increase in 30-s and 50-s ribosomal subunits and no distinct 70-s peak, but the polysomes show

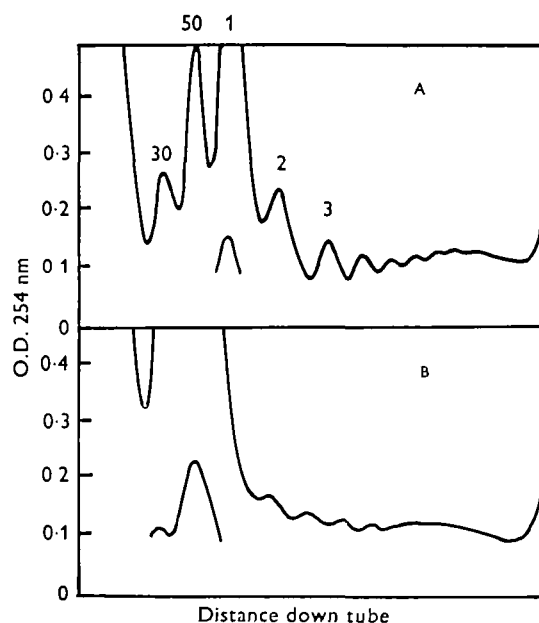


Fig. 1. Sucrose gradient sedimentation pattern of ribosomes and polysomes from uninfected bacteria. Lysates were made and centrifuged for 20 min in gradients containing (A) 0.01 M Mg^{2+} , 0.01 M phosphate or (B) 0.01 M Mg^{2+} , 0.03 M phosphate. The disconnected sections of the tracing are recorded on a 1/5 scale of optical density. Sedimentation is from left to right.

only a slight decline in the largest sizes. When the 70-s ribosomes were taken from a 0.01 M phosphate gradient and re-centrifuged in 0.03 M phosphate (always with 0.01 M Mg^{2+}) they dissociated into 30-s and 50-s subunits. But 70-s ribosomes obtained by treating polysomes with pancreatic ribonuclease (RNase) did not dissociate into subunits under these conditions. This fact reassures us that the 70-s ribosomes found in 0.01 M phosphate are not the result of degradation of polysomes by RNase. They are less stable ribosomes whose dissociation to subunits depends *in vitro*, and presumably also *in vivo*, on the ionic environment.

Fig. 2 shows the distribution of ribosomes from infected spheroplasts in 0.03 M phosphate. The prominent peaks of monomers, dimers and trimers are characteristic,

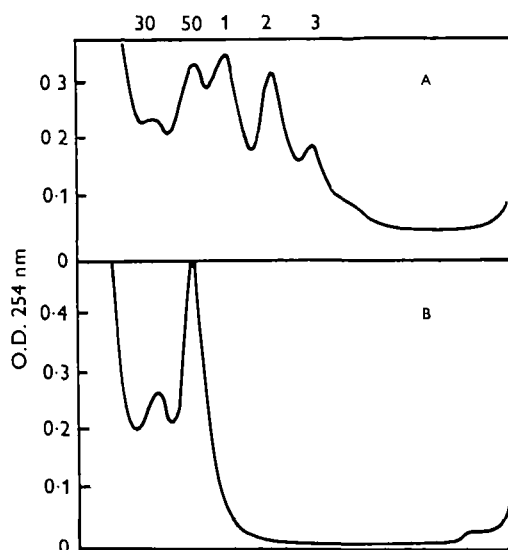


Fig. 2. A, Lysate of infected actinomycin-treated spheroplasts, 40 min after infection. B, Uninfected actinomycin-treated control. Sucrose gradients containing 0.01 M Mg^{2+} , 0.03 M phosphate, were centrifuged for 25 min.

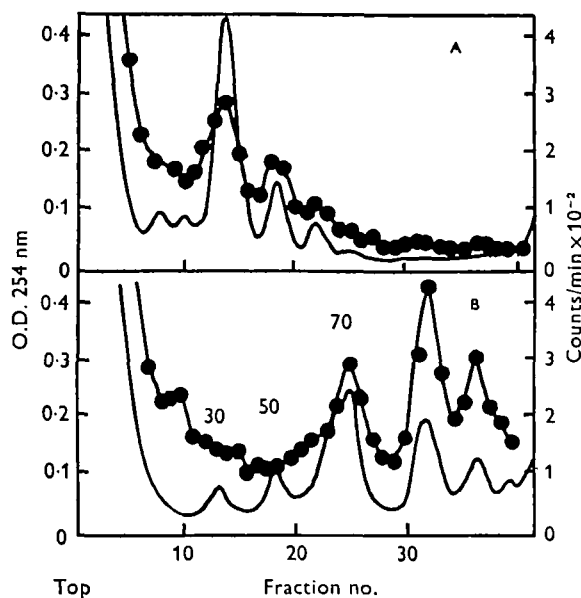


Fig. 3. Lysate of infected spheroplasts labelled with ^{14}C -amino acids ($5\mu Ci/ml$) for 30 s 40 min after infection. Gradients containing 0.01 M Mg^{2+} , 0.01 M phosphate, were centrifuged for (A) 25 min or (B) 45 min. —, optical density; ●—●, ^{14}C .

and are absent from the uninfected actinomycin-treated control (Fig. 2B), in which all the polysomes have been broken down and dissociated into subunits.

Function of polysomes

A brief pulse of radioactive amino acids administered to infected spheroplasts labelled the ribosome monomers, dimers and trimers distinctly (Fig. 3). After the longer centrifugation (Fig. 3B) the 70-s peak of optical density is partly resolved into two. The slower is free 70-s ribosomes (stable in 0.01 M phosphate) and the faster probably contains mature virus as well as viral RNA-ribosomes complexes (see below), with labelled nascent protein attached. The pattern of labelling was unchanged in 0.03 M phosphate although the free (inactive) 70-s ribosomes dissociated into subunits. A control sample was centrifuged after treatment with EDTA and 1% sodium dodecylsulphate to confirm that the amino acid label in the single ribosome region was not in intact phage. All the radioactivity was found at the top of the gradient, and a peak of phage remained, amounting to about 20% of the optical density of the original '70-s' peak.

These results show that in actinomycin-treated infected spheroplasts, in which only virus-specific polysomes exist and only viral proteins are made, the predominant active ribosomes are monomers and dimers, with decreasing numbers of trimers, tetramers, etc.

The following experiments describe the distribution of newly made viral RNA in sucrose gradients and its relationship to ribosomes.

Replicative intermediate

A very short pulse of [³H]uridine labels primarily the partly double-stranded replicative intermediate (RI) RNA in infected actinomycin-treated spheroplasts (M. L. Fenwick, unpublished observations) as in ultraviolet-irradiated cells (Fenwick, Erikson & Franklin, 1964). Fig. 4 shows that after such a pulse the label displays no obvious correlation with the pattern of optical density in a sucrose gradient but is

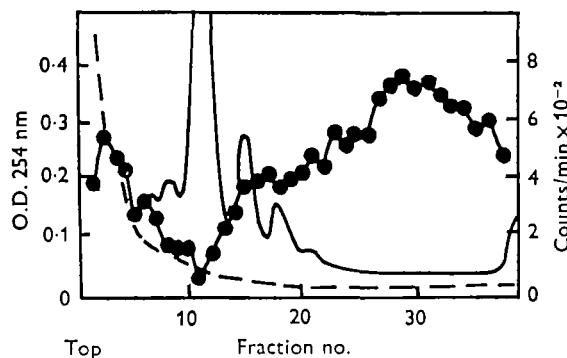


Fig. 4. Replicative intermediate (RI) labelled for 10 s with [³H]uridine 40 min after infection. Sucrose gradient in 0.01 M Mg²⁺, 0.01 M phosphate, centrifuged for 20 min. —, optical density; ●—●, ³H; ---, ³H in an uninfected actinomycin-treated control sample.

distributed broadly with a maximum in the vicinity of the groups of 8–10 ribosomes (such large polysomes are barely detectable by optical density in these infected lysates). This pattern of distribution of RI was essentially stable in 0.03M phosphate, being shifted only slightly to lower sedimentation rates.

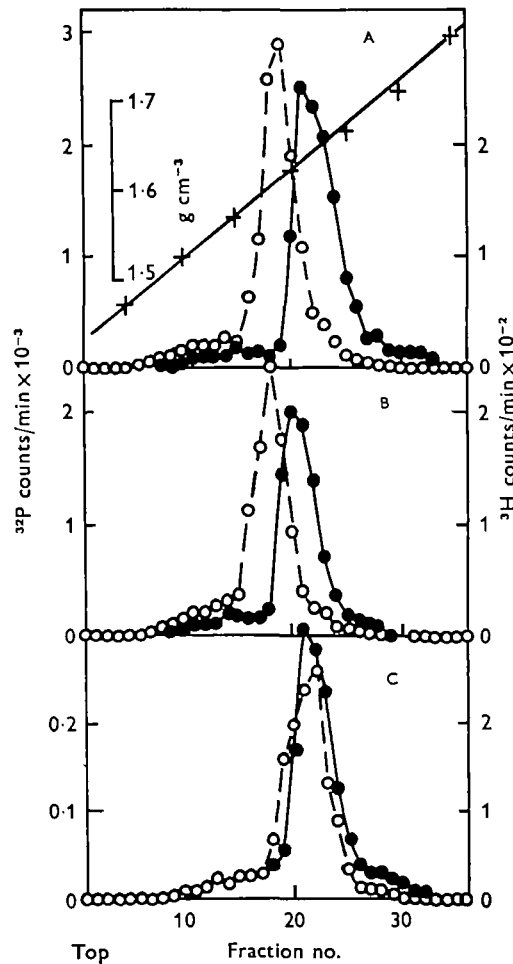


Fig. 5. Buoyant density in CsCl of ^3H -labelled RI taken from (A) the 5–6 ribosome region and (B) the 7–9 ribosome region of a sucrose gradient and mixed, after fixing with formaldehyde, with ^{32}P -labelled 70-s ribosomes from uninfected cells. (C) ^3H -labelled RI and ^{32}P -labelled polysomes obtained from the same infected lysate (5–6 ribosome region). ●—●, ^3H ; ○—○, ^{32}P ; +—+, density of CsCl.

RI sediments at about 16s after extraction with phenol (Fenwick *et al.* 1964). Its fast sedimentation in lysates might be due to aggregation or to association with polysomes as suggested by Hotham-Iglewski & Franklin (1967). Therefore we measured its buoyant density in CsCl. Two fractions of pulse-labelled RI were collected from a gradient such as that of Fig. 4, (i) from the section normally containing groups of 5–6 ribosomes and (ii) from the 7 to 9 ribosome section. These fractions were fixed with formaldehyde and each mixed with a fixed sample of normal 70-s ribosomes isolated

in a similar way from uninfected cells that had been labelled in their ribosomal RNA by growing for 1 h in the presence of [^{32}P]phosphate. The mixtures were then centrifuged in CsCl with the results shown in Fig. 5 A, B. The buoyant density of the RI was about 1.64 g cm^{-3} ($1.64 \times 10^3 \text{ kg m}^{-3}$) and that of the normal 70-s ribosomes 1.61 g cm^{-3} . There was no apparent difference in the density between the slow and fast samples of RI. This density suggested that the RI was associated with light material such as ribosomes or protein, since RI has a density of 1.62 in Cs_2SO_4 (Erikson, 1966) and therefore an expected density of about 1.9 in CsCl.

In order to determine whether the RI was associated with ribosomes, bacteria were pre-labelled by a long incubation with ^{32}P and washed before infection. They were pulse-labelled with [^3H]uridine 40 min after infection, lysed and centrifuged. The section of the gradient corresponding to groups of 5–6 ribosomes was collected, fixed and centrifuged in CsCl (Fig. 5 C). The RI and ribosomal labels were not separated, although the ribosomal ^{32}P shows a slight bias towards lighter density. This bias may be due to a small proportion of polysomal ribosomes bearing intact viral RNA, which would have a density only slightly more than normal 70-s ribosomes (Fenwick, 1971).

These experiments suggest that RI is attached to ribosomes forming a complex which has a density intermediate between those of free ribosomes and free RI.

Newly made viral RNA

When the period of incubation with [^3H]uridine was increased to 2 min or more intact labelled viral RNA strands began to accumulate and the distribution of radioactive RNA in sucrose gradients followed much more closely the optical density pattern, as expected if the viral RNA associates with ribosomes to direct protein synthesis. Fig. 6 shows the distribution of such label, and the effect of 0.03 M phosphate, after a relatively long centrifugation, displaying clearly the ribosomal subunits. A peak of radioactive RNA sediments slightly ahead of the 30-s ribosome subunits (Fig. 6 A), another just ahead of the main 70-s ribosomes and further peaks are associated with the ribosome dimers and trimers. The labelled viral RNA sedimenting at about 75 s is attached to single (70-s) ribosomes (Fenwick, 1971). In a control experiment to determine whether any of the pulse-labelled RNA was within ribosomes or mature virus, an infected lysate was centrifuged in a low magnesium concentration (Fig. 7 A) or after treatment with RNase (Fig. 7 B). In low magnesium the ribosomes all broke down to their constituent 30- and 50-s subunits. Much of the tritium coincided with the 30-s but none with the 50-s subunits, nor with the small peak of mature virus (79-s). All of the labelled RNA was degraded by a low concentration of RNase and was therefore not in intact ribosomes.

Closely similar patterns to that of Fig. 6 A were obtained with 2-min or 5-min labelling periods. Thus there is no evidence that the 30 s-associated label was either a precursor or a metabolic product of the ribosome-associated label. When a lysate was made and centrifuged in the presence of 0.01 M magnesium acetate, 0.03 M sodium phosphate (Fig. 6 B) the optical density tracing showed that many of the 70-s ribosomes had broken down to subunits but the radioactivity pattern was unchanged. Those ribosomes with viral RNA attached were stable and a secondary peak of intact phage

can just be distinguished. The label in the 30-s region now trails slightly behind the enlarged 30-s subunit peak, suggesting that if it was actually attached to subunits in 0.01 M phosphate it became dissociated from them in 0.03 M phosphate. The 30-s region from a gradient with 0.01 M phosphate was fixed and centrifuged in CsCl. The viral RNA label was spread over a wide range of densities between 1.6 and 1.8 g cm⁻³ with a broad maximum at about 1.74 g cm⁻³. This supports the idea that at least part of it was attached to the subunits.

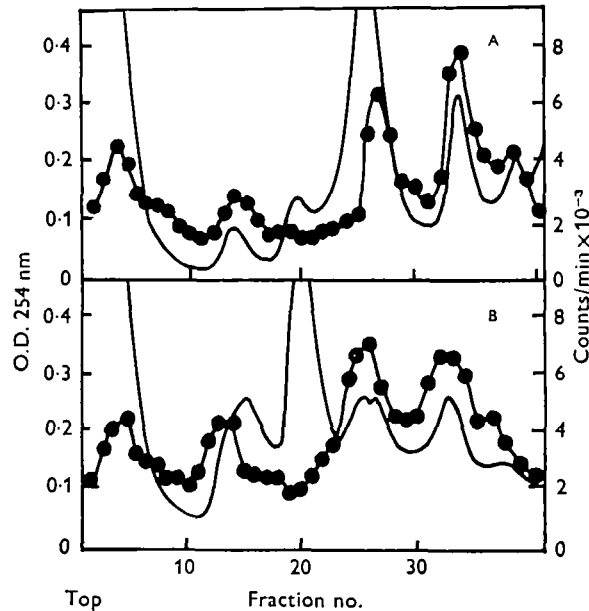


Fig. 6. Completed strands of viral RNA labelled for 10 min with [³H]uridine 30–40 min after infection. Sucrose gradients in (A) 0.01 M Mg²⁺, 0.01 M phosphate, or (B) 0.01 M Mg²⁺, 0.03 M phosphate, were centrifuged for 50 min. —, optical density; ●—●, ³H.

Fig. 8 shows the sedimentation pattern of the RNA extracted from the ribosome dimers obtained from a sucrose gradient such as that of Fig. 6A. The relative areas of the 3 peaks, measured with a planimeter, are small ribosomal (16-s) RNA, 0.53; large ribosomal (23-s) RNA, 1.00; phage (27-s) RNA, 0.33. The radioactivity formed a peak at 27s which contained 50% of the counts. The remaining 50% were distributed over a range of from 27s to about 12s. The same pattern of radioactivity was found on extracting the RNA from each individual polysome peak from the 1's to the 6's and also from the 30-s subunit region. In each case between 45 and 50% of the tritium was found in the 27-s peak. The optical densities obtained from the larger polysomes were too low for accurate measurement and that from the singles was of little significance because of the presence, in 0.01 M phosphate, of excess ribosomes unattached to viral RNA (Fenwick, 1971) and of mature virus.

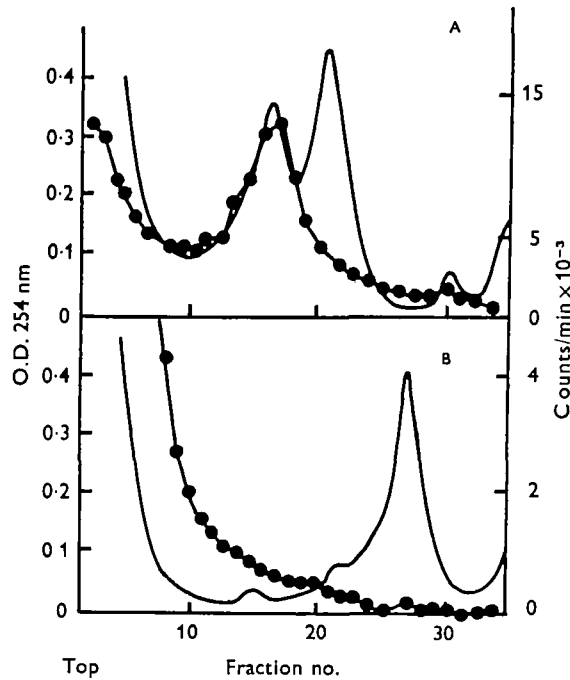


Fig. 7. Effect of low Mg²⁺ and RNase on newly made viral RNA. Spheroplasts were labelled with [³H]uridine 30–40 min after infection. A, Lysed and centrifuged for 90 min in 10⁻⁵ M Mg²⁺, 0.01 M tris pH 7.2. B, Lysed in 0.01 M Mg²⁺, 0.01 M phosphate, treated with RNase (0.01 μg/ml for 10 min at 20 °C) and centrifuged in the same medium for 75 min. —, optical density; ●—●, ³H.

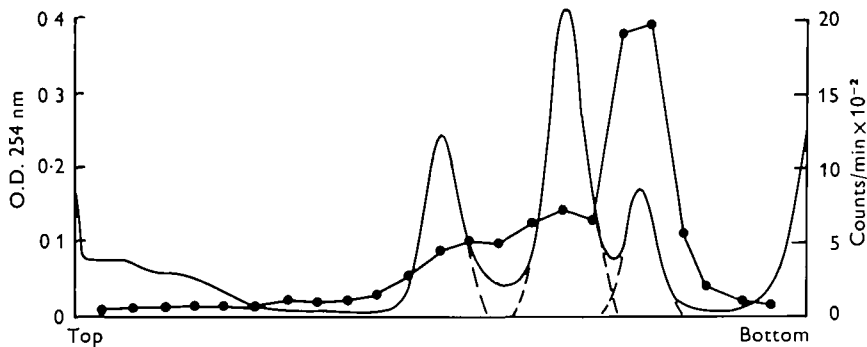


Fig. 8. RNA extracted from infected ribosome dimers. The dimer peak was collected from a sucrose gradient like that of Fig. 6 A and RNA extracted from it with phenol in the presence of 0.02 M EDTA and 1% sodium dodecylsulphate, and precipitated with ethanol. The RNA was dissolved and centrifuged in a second sucrose gradient in 0.1 M NaCl, 0.01 M tris at 50000 rev/min for 2.5 h at 20 °C. —, optical density; ●—●, ³H.

Kinetics of incorporation of new viral RNA into phage

As mentioned before, the great majority of the labelled viral RNA after a 10-min pulse of tritiated uridine is found associated with ribosomes and not in progeny phage. This is so if the pulse is administered at any time between 30 and 60 min after infection (40–50 min being the time of maximum infective virus production).

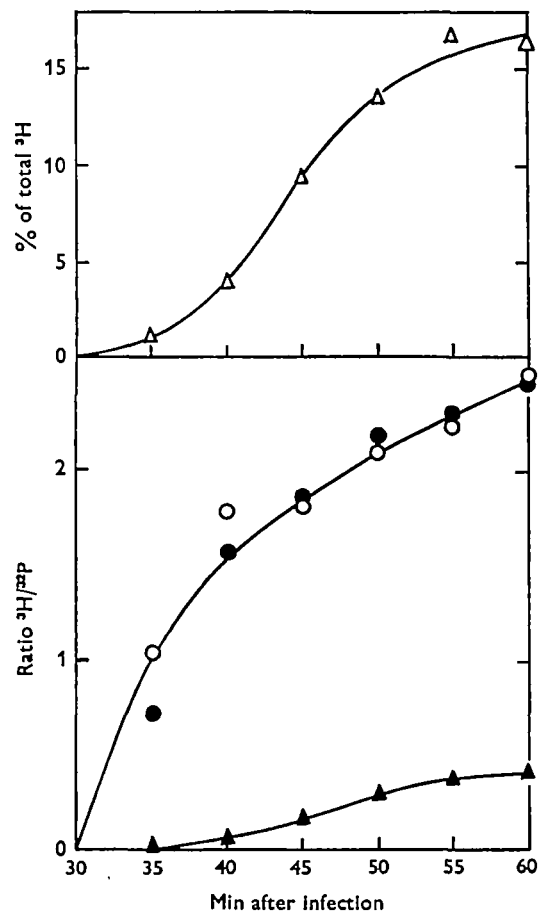


Fig. 9. Accumulation of ^3H -viral RNA in polysomes and in mature phage with continuous labelling. The cells were pre-labelled with ^{32}P in their ribosomal RNA before infection. \circ — \circ , average polysomal $^3\text{H}/^{32}\text{P}$; \bullet — \bullet , total $^3\text{H}/\text{total } ^{32}\text{P}$; \blacktriangle — \blacktriangle , phage $^3\text{H}/\text{total } ^{32}\text{P}$; \triangle — \triangle , phage $^3\text{H} \times 100/\text{total } ^3\text{H}$ (see text).

In order to follow the incorporation of labelled RNA into phage, bacteria were pre-labelled with ^{32}P in their ribosomal RNA. Tritiated uridine was added to the spheroplasts 30 min after infection and samples taken at 5-min intervals. Each sample was lysed and analysed on a sucrose gradient. The ^{32}P recovery permitted correction for variations in yield of total ribosomal material from different samples. Total viral RNA was obtained by adding all the tritium counts beyond the peak of transfer RNA and fragments at the top of the tube. Total ^{32}P was calculated similarly. The ratio of

polysomal ^3H to ^{32}P is the average of the $^3\text{H}/^{32}\text{P}$ ratio in all fractions from the groups of 2, 3 and 4 ribosomes. Intact phage was determined by treating half of each fraction in the 70-s region with RNase before precipitating with TCA and counting. All the ribosome- and polysome-associated viral RNA is degraded by RNase.

It can be seen in Fig. 9 that tritium began to accumulate in mature phage after a lag of about 5 min and reached a level of 16% of the total labelled viral RNA by the end of the growth cycle.

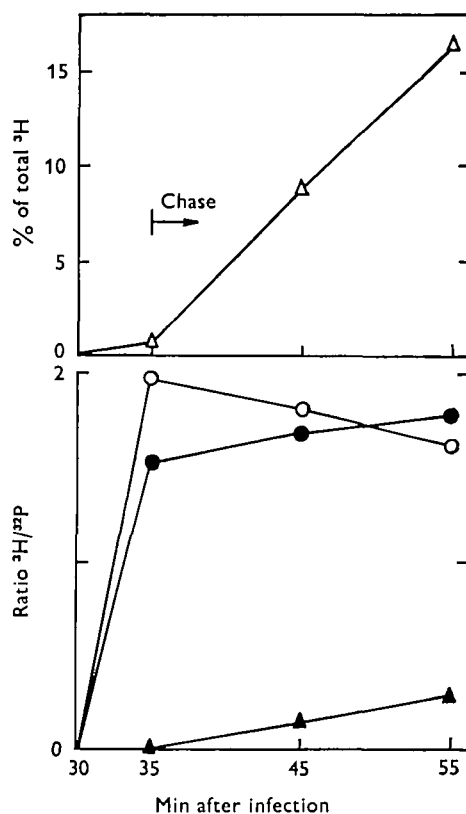


Fig. 10. Fate of viral RNA during a chase. After labelling with [^3H]uridine from 30 to 35 min after infection spheroplasts were resuspended in non-radioactive medium. ○—○, average polysomal $^3\text{H}/^{32}\text{P}$; ●—●, total $^3\text{H}/\text{total } ^{32}\text{P}$; ▲—▲, phage $^3\text{H}/\text{total } ^{32}\text{P}$; △—△, phage $^3\text{H} \times 100/\text{total } ^3\text{H}$.

In order to determine whether the RNA which associates with ribosomes can be later transferred to progeny virus, after a 5-min pulse of tritiated uridine ($1\mu\text{Ci}/\text{ml}$) the spheroplasts were poured on to an equal volume of frozen medium containing uridine, $10\mu\text{g}/\text{ml}$, centrifuged and resuspended in non-radioactive medium containing actinomycin and uridine, $10\mu\text{g}/\text{ml}$. Samples were analysed as before after 10- or 20-min chase periods. Fig. 10 shows that although the specific radioactivity of the polysomes fell slightly during the chase, label accumulated in phage in much the same way (expressed as a percentage of the total labelled viral RNA) as in the previous experiment with continuous labelling. Thus a phage precursor had been labelled during the first

5 min and the results are consistent with the idea that about 16% of the viral RNA that initially attaches to ribosomes is destined to appear subsequently in phage while the majority remains in a relatively stable association.

DISCUSSION

Although it is generally agreed that polysomes consist of groups of ribosomes engaged in protein synthesis, there is some controversy over the significance of free 70-s ribosomes in bacterial lysates. Under certain conditions few or no 70-s ribosomes are found (Mangiarotti & Schlessinger, 1966; Phillips, Hotham-Iglewski & Franklin, 1969*a, b*; Varrichio, 1969) and it has been suggested that the appearance of a 70-s peak indicates either the degradation of polysomes or the artificial aggregation of ribosomal subunits. On the other hand it has been claimed (Kohler, Ron & Davis, 1968; Algranati, Gonzales & Bade, 1969) that 70-s ribosomes are a natural end-product of protein synthesis in polysomes and that a specific dissociation factor is required to separate the subunits before starting a fresh cycle of synthesis (Subramanian, Ron & Davis, 1968). It was shown that these free ribosomes are less stable in low concentrations of magnesium than protein-synthesizing ribosomes (Ron, Kohler & Davis, 1968) or than ribosomes obtained by ribonuclease treatment of polysomes. As described here, a similar distinction can be made by increasing the sodium phosphate concentration: most of the 70-s ribosomes dissociate into subunits at a concentration which has little effect on polysomes. It is possible to choose a medium that will give any desired ratio of intact to dissociated single ribosomes. Which are the natural immediate products of protein synthesis is still not settled, but it is clear that in uninfected lysates only a small minority of the stable ribosomes are single, i.e. monosomes. With the cells and conditions used here an optical density maximum was observed in sucrose gradients in the region of groups of about 8 ribosomes.

Infection with the RNA phage R17 causes a loss of the larger polysomes and accumulation of the smaller ones and stable 70-s ribosomes (Hotham-Iglewski & Franklin, 1967; Hotham-Iglewski *et al.* 1968) and Godson (1968) was able to detect infective viral RNA in phenol extracts of the small polysomes. It was not clear which or how many of these were involved in making viral protein and which merely degradation products of host polysomes. It was suggested that the 70-s ribosomes may be ribosome-cellular mRNA complexes with a block in polypeptide chain growth (Phillips, Truden, Iglewski, Hotham-Iglewski & Franklin, 1969). In infected spheroplasts treated with actinomycin the situation is clearer since actinomycin alone causes the total loss of polysomes and stable monosomes with concomitant cessation of cellular protein synthesis. The prominent small polysomes and stable monosomes are therefore virus-specific. Furthermore, they have been shown to carry intact viral RNA and to be making protein. The reason why such a large mRNA as the phage RNA should form such small polysomes is not clear. It may be related to the high degree of secondary structure in the phage RNA (Fenwick, 1968*b*), to the unhealthy metabolic state of the host cell, or simply to the ratio of phage RNA molecules to available ribosomes.

The distribution of pulse-labelled RNA amongst the larger polysomes in our

sucrose gradients agrees with the findings of others (Hotham-Iglewski & Franklin, 1967; Hotham-Iglewski *et al.* 1968; Godson, 1968) who used other techniques to detect RI. They (Hotham-Iglewski & Franklin, 1967) showed that RI sedimented much more slowly in a magnesium concentration low enough to disaggregate the polysomes and suggested that it was actually attached to ribosomes. Our demonstration that the RI has the same buoyant density as ribosomal material that cosediments with it supports this suggestion. It is possible to make estimates of the expected buoyant densities of various hypothetical RI-ribosome complexes, making the following assumptions: RI has an average molecular weight of about 3×10^6 (2 complete strands of 1.1×10^6 (Gesteland & Boedtke, 1964) together with an uncertain number of nascent strands) and a density of 1.9 g cm^{-3} in CsCl (estimated from its density of 1.62 in Cs_2SO_4 (Erikson, 1966)); ribosomes have a molecular weight of 2.7×10^8 (Petermann, 1964) and a density (this paper) of 1.61 g cm^{-3} . The estimated densities, in g cm^{-3} , are: 1 RI + 2 ribosomes 1.70 ; 1 RI + 6 ribosomes 1.65 ; and 1 RI + 10 ribosomes 1.63 .

We observed a density of 1.64 using RI from the vicinity of the groups of 5–9 ribosomes, which is consistent with the existence of the proposed complex. It would be anticipated that the slowest sedimenting RI, from the ribosome dimer region, would have a higher density.

The RNA extracted from the ribosome dimers after a 10-min labelling period and analysed on a sucrose gradient showed that about 50% of the labelled RNA was intact (27-s) viral RNA. The optical density tracing showed a peak at 27s whose area was 33% of that of the 23-s ribosomal RNA peak. Since the two RNA's have approximately the same molecular weight, the ratio of intact viral RNA to ribosomal RNA molecules was also 0.33 to 1. This value does not take into account the degraded or incomplete viral RNA revealed by the radioactivity and so is not inconsistent with the expected ratio of 0.5 viral RNA molecules per ribosome.

The peak of new viral RNA sedimenting in the 30-s region is probably attached to ribosomal subunits. A density of about 1.74 would be predicted for such a complex although the observed wide range of higher densities would suggest that the binding is weak or incomplete. The viral RNA sedimenting at about 75s is probably attached to single (70-s) ribosomes as discussed in the following paper (Fenwick, 1971). In terms of radioactivity, this is the most prominent peak after a 10-min labelling period, although the actual number of ribosomes involved is lower than in the ribosome dimers. The 75-s complex could easily be confused with intact (79-s) virus, but can be distinguished from the latter by its sensitivity to low magnesium and ribonuclease. Webster & Zinder (1969) observed a similar (80-s) structure bearing viral RNA after adding RNA to ribosomes from *E. coli* in a medium allowing initiation of protein synthesis. In studies of the fate of radioactive parental phage RNA, Godson & Sinsheimer (1967) found a labelled peak just ahead of the 70-s ribosomes in sucrose gradients, which was presumed to be intact phage since its sedimentation rate was not lowered in 10^{-4} M MgCl_2 .

The kinetic experiments show that the viral mRNA in polysomes is stable and that the incorporation of RNA into mature virus particles is inefficient. Most, if not all, of the newly made RNA associates with ribosomes, probably even before its release

from RI. The small decline in labelled polysomal viral RNA during a chase was similar in amount to the label accumulating in virions, suggesting that there is some channelling of the polysome-bound RNA into virions. The possibility that about 16% of the new viral RNA embarks on a separate pathway leading to virions cannot be excluded, but it does not form a distinct peak in sucrose gradients. The only non-ribosome-bound peak after a 5-min labelling period is in the 30-s region, and this did not decline during a chase and is therefore not a specific virus precursor. Davis & Sinsheimer (1963) found less than 3% transfer of adsorbed parental RNA to progeny phage. The difference between this figure and our observation that the transfer to progeny phage of RNA made between 30 and 35 min after infection is limited to about 16% may well be explained by the high risk of degradation of entering strands during the first 30 min (Engelberg & Artmann, 1970).

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