SIZE DISTRIBUTION OF "INFORMATIONAL" RNA

B. P. SAGIK, M. H. GREEN, M. HAYASHI, *and* S. SPIEGELMAN
From the Departments of Microbiology and Chemistry, University of Illinois, Urbana

ABSTRACT Previous investigations suggested that the size of "informational" or "messenger" RNA was confined to sedimentation rates lying between 8 and 14S. These involved procedures permitting extended contact of the RNA with enzymatically active extracts. The present study re-examined the size distribution of T2-complementary RNA isolated by a method which minimized enzymatic degradation. A much greater diversity in size distribution (4S to 25S) was observed. Experiments are described indicating that 8 to 12S informational RNA does not readily attach to the 16S and 23S ribosomal components under the conditions used for sedimentation analysis.

INTRODUCTION

Data suggesting the existence of "informational"[1] ribonucleic acid came initially from experiments by Volkin and Astrachan (1) in which T2-infected *Escherichia coli* cells were pulsed with P^{32}. An RNA was detected possessing an apparent base composition analogous to T2 DNA. Using the same system, Nomura, Hall, and Spiegelman (2) physically separated the newly synthesized RNA from the bulk of the cellular RNA and thus established the existence of informational RNA as a distinct physical entity. Hall and Spiegelman (3) showed that T2-complementary RNA would specifically hybridize with heat-denatured T2 DNA. It was concluded that the homology in base composition extended to a detailed complementary correspondence in base sequence. Using the same procedures, a similar sequence homology test was carried out successfully by Geiduschek, Nakamoto and Weiss (4) with RNA synthesized enzymatically, using T2 DNA as the template.

For the present purposes, it is pertinent to note a feature of the methods used

[1] We will use in the present discussion, as we have in previous ones, the terms "informational" or "complementary" RNA to designate a population of RNA molecules possessing an over-all base composition analogous to its homologous desoxyribonucleic acid and capable of specifically hybridizing with it. Within this class we may presume will be found the "messenger" RNA postulated by Jacob and Monod (5) on theoretical grounds to constitute the programs of protein biosynthesis.

for the selective enrichment of informational RNA in the earlier investigations (2, 3). They involved preliminary separation of the ribosomes by the usual 2 to 3 hour centrifugations at 40,000 RPM. A determination of the size distribution of the labeled RNA found with the ribosomes of T2-infected cells revealed a rather broad distribution centering between 8 and 14S. Other laboratories, seeking to extend these observations to uninfected cells, used the same methods of separation and detected (6-8) the existence of a rapidly labeled RNA in about the same size range (8 to 14S). In addition, the complementary RNA synthesized by the DNA-dependent RNA polymerase was found (4) to have an average sedimentation constant of about 6S.

In view of these observations, it is perhaps understandable that references in the recent literature to messenger RNA have quite unanimously concluded that a distinct average size (8 to 14S) is a characteristic which serves to distinguish this RNA from the three major components of the cell (23S, 16S, and 4S). There are, however, reasons to doubt whether this is in fact the case. As our own experience with complementary RNA accumulated, it became evident that this type of RNA is uniquely susceptible to degradation in extracts. It seemed possible that the small size observed in the earlier experiments might be an artifact due to breakdown in the course of preparation. The methods for the isolation of complementary RNA were changed accordingly. Such modifications were employed in the study of the RNA preferentially synthesized during a step-down transition from fast to slow growth (9). Here, a much wider size distribution of complementary RNA was observed. In view of these findings, a reinvestigation of the size of T2 RNA was initiated. It is the purpose of the present paper to present the data obtained. They indicate that the use of preparative procedures which avoid prolonged contact with enzymatically active extracts leads to the detection of informational RNA of extremely diverse sizes extending from above 23S to 4S.

MATERIALS AND METHODS

(*a*) *Cells and Infection.* *E. coli* strains (B and K-16) and bacteriophage T2 were used. Cells were grown overnight on a rotary shaker at 37°C in synthetic C medium (10) (buffered with 0.1 M phosphate, pH 7.1). The cells of an overnight culture were collected by centrifugation and resuspended in fresh medium at an $OD_{660} \cong 0.150$ (*ca.* 5×10^8 cells/ml). The suspension was then reincubated at 37°C until the titer reached 1×10^9 cells/ml, $OD_{660} \cong 0.300$. After centrifugation, the cells were resuspended in fresh medium in 1 per cent of the original volume, *i.e.* 1×10^{11} cells/ml, cooled to 6-7°C, and infected with bacteriophage at a multiplicity of about 10 phages per cell. After 15 minutes the infected cells were pipetted into vigorously aerated medium at 37°C. The dilution was such that the final cell concentration was 1×10^9 per ml. Viable cell counts were performed at various times subsequent to infection. The time course of infection for a typical experiment is given in Table I.

TABLE I

Time	Uninfected cells
min.	*per cent*
0	3.5
2.5	0.08
5	0.005

(*b*) *Pulse Experiments.* H^3-uridine (0.2 $\mu c/\mu g$/ml) or 2-C^{14} uracil (0.06 $\mu c/\mu g$/ml) was added to aliquots of the phage-infected bacteria at various times after infection. At the desired times after the addition of these labeled RNA precursors, the pulse was stopped by adding the infected cells to an equal volume of finely ground ice (C medium at −70°). The cells were centrifuged at 5°C and resuspended in 2 ml of cold tris (0.01 M, pH 7.3)–KCl (0.01 M)–$MgCl_2$ (0.005 M) solution, containing lysozyme (300 μg/ml) and DNAase (50 μg/ml). They were then frozen rapidly in methanol-CO_2, thawed and held 5 minutes at 14°C. The solution was then brought to 1 per cent in sodium dodecyl sulfate and shaken for 5 minutes at 20°C. RNA was isolated by the phenol method of Gierer and Schramm (11).

(*c*) *Sedimentation Analysis.* Ultracentrifugal examination of the RNA was carried out as described previously (3) using a swinging bucket rotor, Spinco type SW-25 in the model L centrifuge. The RNA solutions were placed in the centrifuge tube in the form of a layer (2 ml volume) on top of 28 ml of a linear sucrose gradient (2.5 to 15 per cent by weight). The general buffer used was 0.01 M tris(hydroxymethyl) amino methane (tris) buffered at pH 7.4 and containing 0.005 M $MgCl_2$. In certain instances to be noted the $MgCl_2$ was omitted. Centrifugation was at 25,000 RPM at 5°C for 11.5 hours. Fractions were collected, after puncturing the bottom of the tube, and analyzed for ultraviolet absorption and TCA-precipitable radioactivity by methods detailed elsewhere (3).

(*d*) *DNA-RNA Hybrid Formation.* Samples of RNA from various positions in the sucrose density gradient runs were used for forming hybrids. The RNA was dialyzed against 0.3 M NaCl, 0.03 M Na citrate (2 × SSC) prior to incubation with T2 DNA. The T2 DNA was prepared by the method of Grossman, *et al.* (12) and denatured by heating for 15 minutes at 98-100°C at a concentration of 120 μg/ml in 1 × SSC, followed by rapid cooling to 0°C. Mixtures containing equal amounts of labeled RNA and 24 μg/ml heat-denatured DNA were brought to 2 × SSC and incubated for 20 hours at 40°C. After the incubation, they were rapidly cooled to 25°C, saturated with CsCl, and layered below a solution of CsCl having a density of 1.73 gm/cc. Equilibrium density gradient centrifugation was then performed at 33,000 RPM for 2½ to 3 days at 23-25°C in the SW-39 rotor using the Spinco model L ultracentrifuge. Fractions were collected, after puncturing the bottom of the tubes, and analyzed for optical density at 260 mμ and radioactivity as described in section *c* (above).

(*e*) *Step-Down Transitions.* Cultures in "step-down" transitions were obtained as described by Hayashi and Spiegelman (9). *E. coli* growing logarithmically in modified (2) Pen-Assay medium are harvested, washed and resuspended in synthetic medium C. Pulsing was done with H^3-uridine (1600 μc/m mole) for the periods indicated. The RNA was isolated by the procedure described in section *c* (above).

RESULTS

The procedure employed in the present investigation for the isolation of the RNA for subsequent analysis was designed to avoid prolonged exposure to enzymatically active extracts. In principle it depends on cell rupture at low temperatures by freezing and thawing in the presence of lysozyme and DNAase. This is followed by a short incubation at below room temperatures (14-15°) to complete the degradation of DNA. The proteins are then denatured by the addition of detergent and, subsequently, phenol resulting in the complete elimination of RNAase and DNAase activity.

(*a*) *Distribution of Sedimentation Rates.* The sedimentation rates of RNA synthesized during various periods of infection and isolated by the procedures described are shown in Fig. 1. The optical density profiles identify the positions of the known RNA components. The first peak corresponds to the 23S and the second to the 16S ribosomal variety. It is apparent from the distribution of radioactivity that the RNA synthesized subsequent to infection shows a much greater diversity in size when isolated by the procedures employed here than was observed in earlier investigations (2, 3, 6-8). Instead of the previously obtained uniform broad peak centering somewhere between 14 and 4S, one notes here a wide distribution of labeled RNA components extending from about 30S down to 4S. Indeed, in the experiments described in Fig. 1 and in others not detailed, it is regularly found that from 43 to 53 per cent of the newly synthesized RNA possesses a sedimentation constant of 16S and greater.

(*b*) *Hybridizability of the Various RNA Sizes.* Although unlikely, one might perhaps entertain the possibility that the isolation procedure preserves large RNA molecules which are not complementary to T2 DNA and, therefore, irrelevant. At present, the most sensitive and conclusive test for complementarity would appear to be hybridizability with homologous DNA (3). Accordingly, RNA fractions from the various size ranges observed in the pulse experiments of Fig. 1 were individually tested for their ability to combine with denatured T2 DNA.

The results of these hybridization experiments are summarized in Figs. 2 and 3. In every instance, DNA-RNA hybrids were readily detected by the appearance of H^3 RNA in the DNA region of the CsCl density gradient. Further, for all the sizes tested, significant proportions (15 to 40 per cent) of the input RNA were complexed to DNA.

As a specificity control for the hybridizability of the larger complementary RNA molecules, it was decided to test the ability of host ribosomal RNA to hybridize with T2 DNA. Accordingly, P^{32}-labeled RNA of the 23S and 16S varieties were prepared from non-infected cells. Fig. 4 shows the outcome of a hybridization test which, as may be seen, is completely negative.

The experiments described thus far would appear to establish that, within the

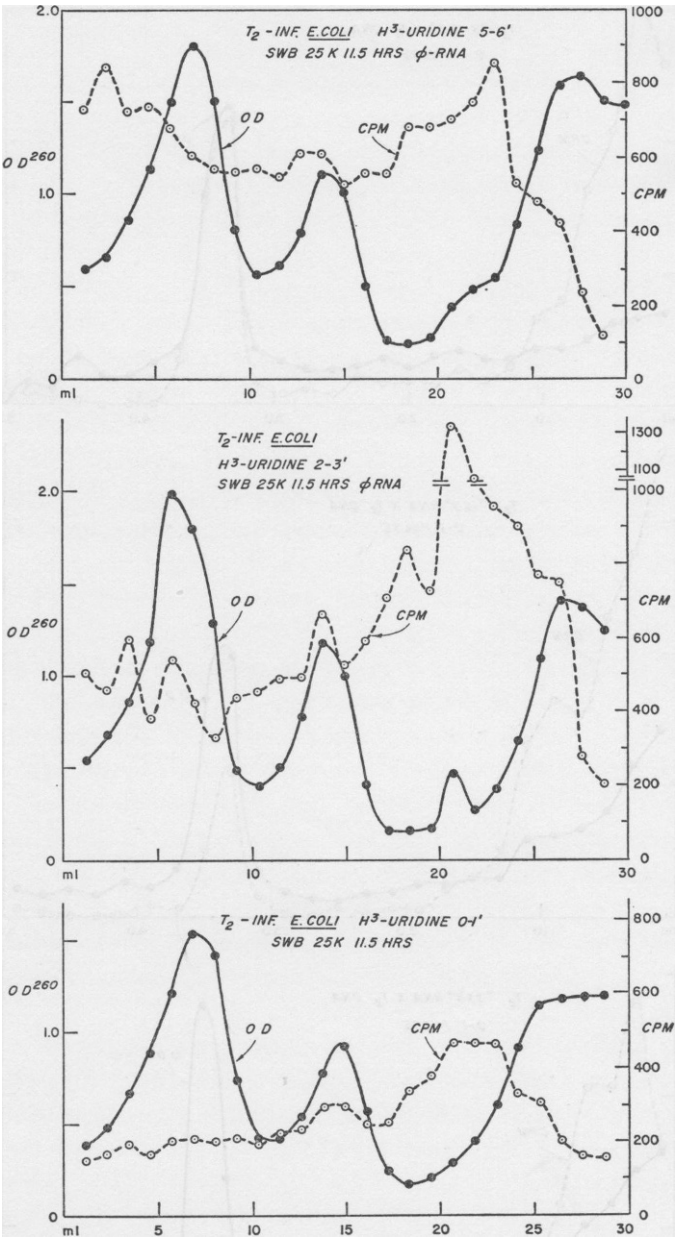

FIGURE 1 *Sedimentation Analysis of RNA.*
Separate aliquots of T2-infected *E. coli* were pulsed with H^3-uridine from 0 to 1, 2 to 3, and 5 to 6 minutes after infection. RNA, isolated by phenol extraction of whole cell extracts, was immediately subjected to sucrose density gradient centrifugation for 11.5 hours at 25,000 RPM. Fractions were collected from the bottom of the centrifuge tube (0 ml) and analyzed as described in the Methods section.

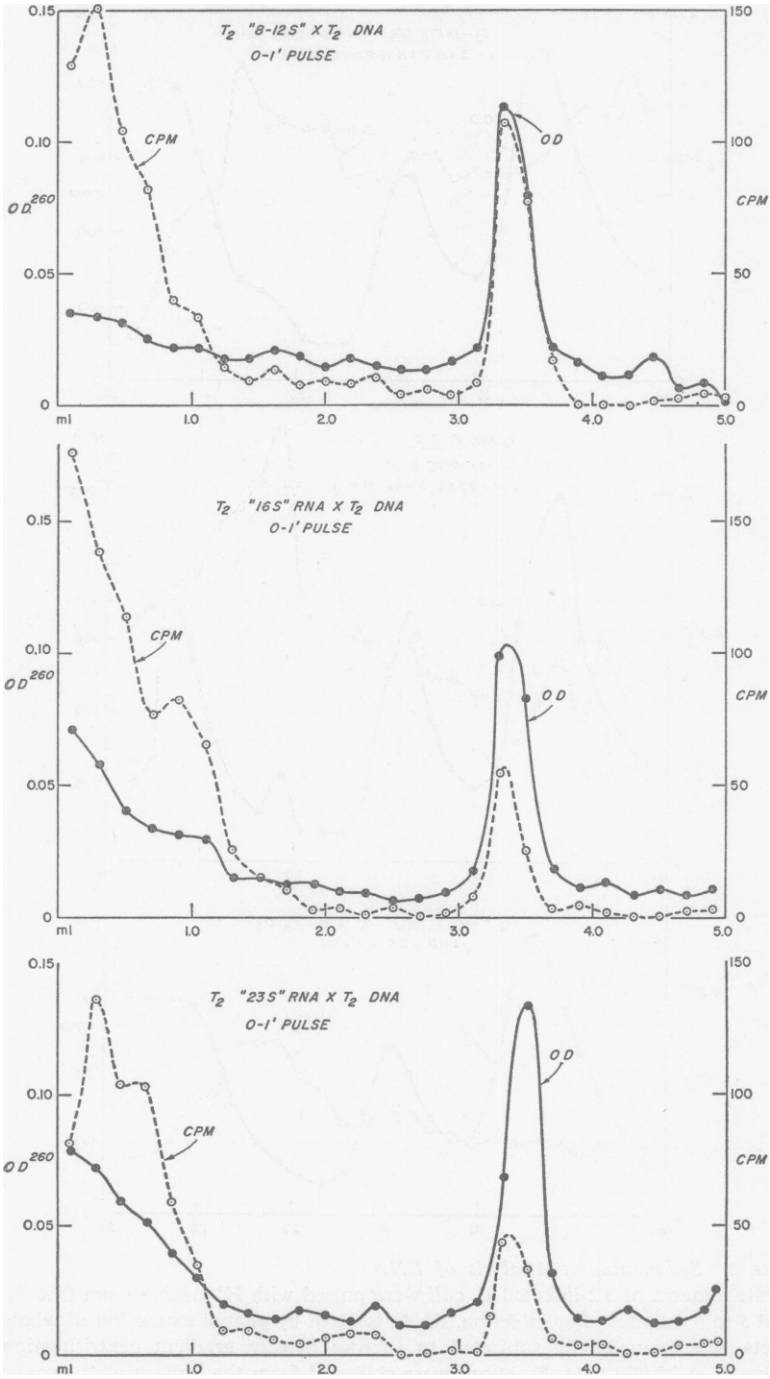
T2 "8-12S" X T2 DNA
0-1' PULSE
CPM
OD
OD260
CPM
ml
T2 "16S" RNA X T2 DNA
0-1' PULSE
T2 "23S" RNA X T2 DNA
0-1' PULSE

FIGURE 2

accuracy assignable to the hybridizing test, the various sizes of RNA molecules synthesized in a T2-infected cell are equivalently complementary to T2 DNA.

(*c*) *Problem of Aggregation with Ribosomal RNA.* One might grant that the labeled RNA molecules observed in the regions above 16S are T2-specific and yet question whether their presence might reflect, not a larger size, but rather an adherence to ribosomal RNA. This possibility is perhaps made even more pertinent by the presence in the sucrose gradients of $MgCl_2$ at a level (0.005 M) which might perhaps encourage aggregation. The fact that the radioactivity and optical density profiles of Fig. 1 coincide so poorly argues against extensive aggregation between the two types of RNA. Nevertheless, it was considered worthwhile to subject this possibility to a more direct test with the aid of two sorts of experiments. In one, the effect of Mg^{++} on the comparative distribution of ribosomal and complementary RNA was examined. In the other, the apparent size distribution of previously isolated 8 to 12S complementary RNA was compared when rerun in the presence and absence of added ribosomal RNA and at different levels of magnesium.

To perform the first type of experiment, RNA from a 3 to 7 minute pulse with 2-C^{14} uracil was prepared from T2-infected cells as detailed above, except that Mg^{++} was omitted. An aliquot of this Mg^{++}-free RNA was dialyzed for 4 hours at 5°C against a 500-fold volume of tris, 0.01 M $MgCl_2$, with one change after 2 hours. These two RNA preparations were then subjected to sucrose density gradient sedimentation analysis, the sucrose gradients containing the same salt concentrations as the RNA solutions. The results are shown in Fig. 5.

It is evident that the omission of Mg^{++} has had comparatively little effect on the size distribution of the complementary RNA relative to the ribosomal components. In the absence of Mg^{++}, 45 per cent of the C^{14}-labeled RNA traveled at least as fast as the 16S ribosomal RNA component, and one finds the usual components possessing sedimentation coefficients of 23S and greater. In the presence of 0.01 M Mg^{++}, 56 per cent of the T2 RNA is found in the regions beyond and including the 16S. It should be noted from a comparison of Figs. 5*a* and 5*b* that the presence of Mg^{++} tends to raise sedimentation constants and magnify differences. The resulting increase in resolution was the original reason for its routine inclusion in sedimentation analyses.

For purposes of comparison with other studies (6, 8), normal informational RNA molecules from uninfected *E. coli* were used in the experiments to test

FIGURE 2 *DNA-RNA Hybrid Formation.*
RNA from the 0 to 1 minute pulse experiment was isolated from the various designated regions of the sucrose gradient (see Fig. 1) and tested for its ability to combine with non-labeled heat-denatured T2 DNA. The profiles show the separation of free RNA from DNA-RNA hybrid after CsCl equilibrium density gradient centrifugation. The highest density is at 0 ml.

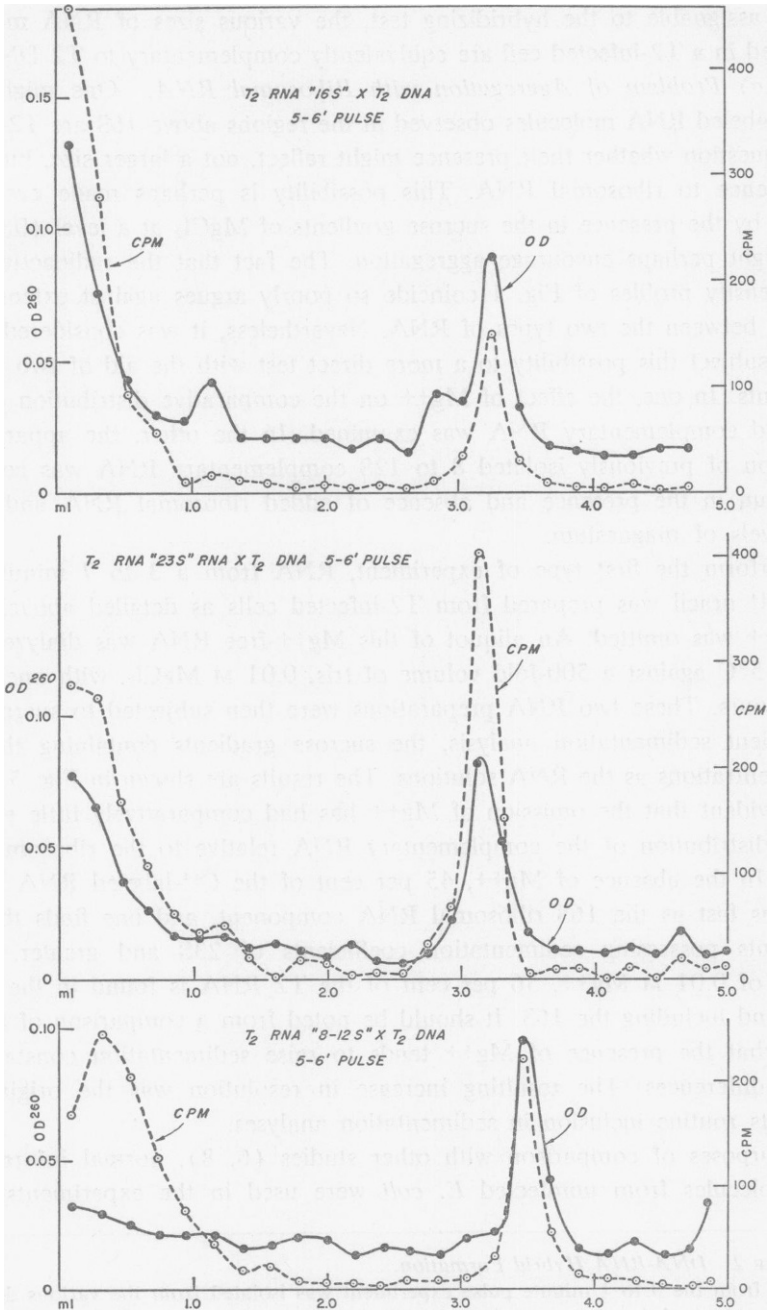

FIGURE 3 *DNA-RNA Hybrid Formation.*
RNA from the 5 to 6 minute pulse experiment was isolated and treated as described in Fig. 2.

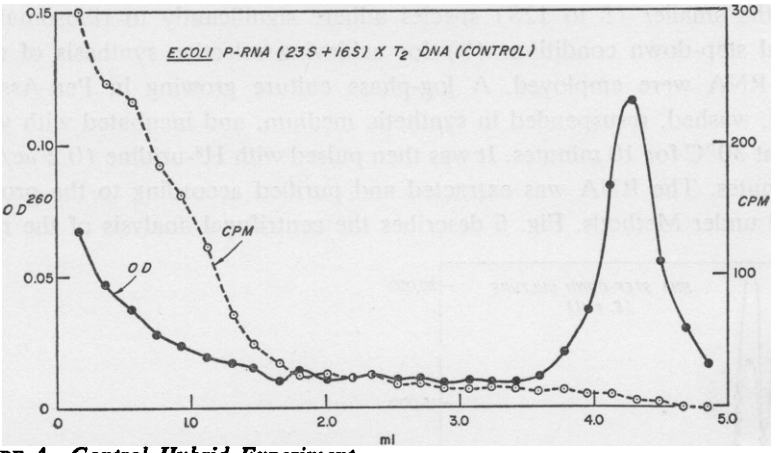

FIGURE 4 *Control Hybrid Experiment.*
P^{32} RNA from non-infected *E. coli* incubated with heat-denatured T2 DNA and treated as described in Fig. 2.

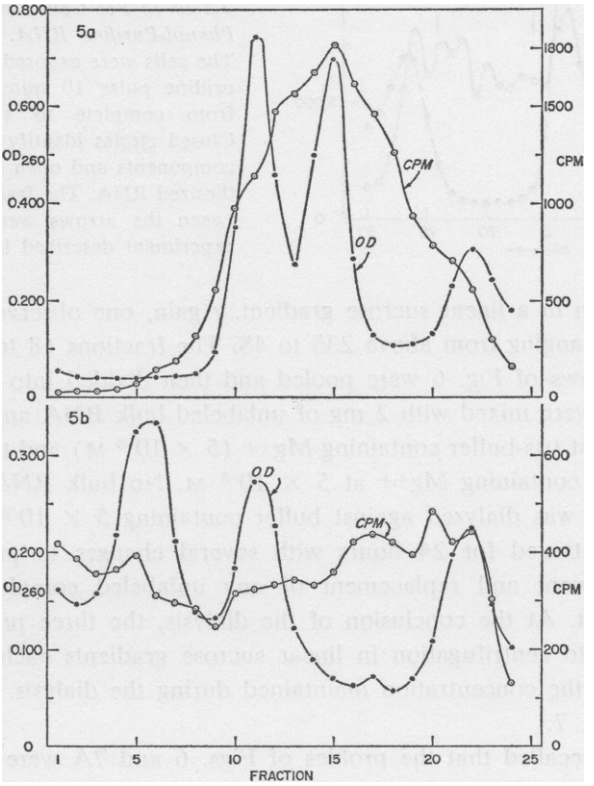

FIGURE 5 *Effect of Mg^{++} on Sedimentation Distribution of T2-Specific RNA*
(*a*) Sucrose gradient centrifugation of Mg^{++}-free RNA.
(*b*) Sucrose gradient centrifugation of RNA in 0.01 M $MgCl_2$.

whether the smaller (8 to 12S) species adhere significantly to ribosomal RNA. The usual step-down conditions (9) for achieving selective synthesis of complementary RNA were employed. A log-phase culture growing in Pen-Assay was harvested, washed, resuspended in synthetic medium, and incubated with vigorous aeration at 30°C for 10 minutes. It was then pulsed with H^3-uridine (0.2 $\mu c/\mu g$/ml) for 3 minutes. The RNA was extracted and purified according to the procedures described under Methods. Fig. 6 describes the centrifugal analysis of the resulting

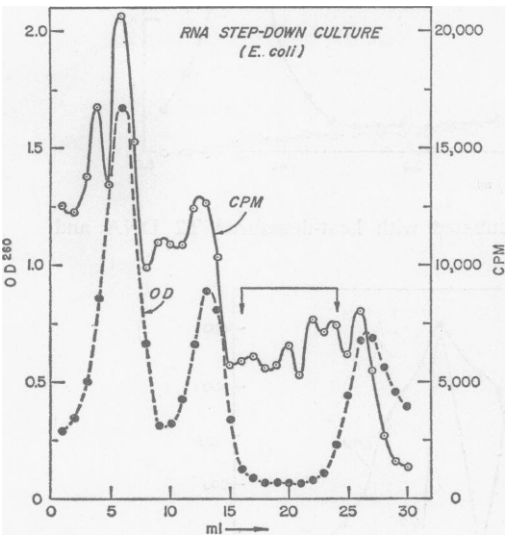

FIGURE 6 *Swinging-Bucket Analysis in 2.5 to 15 Per Cent Sucrose Gradients of Phenol-Purified RNA.*
The cells were exposed to a 3 minute H^3-uridine pulse 10 minutes after transfer from complete to synthetic medium. Closed circles identify pre-existent RNA components and open circles, newly synthesized RNA. The fractions included between the arrows were pooled for the experiment described in Fig. 7.

RNA preparation in a linear sucrose gradient. Again, one observes labeled RNA of diverse sizes ranging from above 23S to 4S. The fractions (8 to 12S) included between the arrows of Fig. 6 were pooled and then divided into 3 aliquots. Aliquots A and B were mixed with 2 mg of unlabeled bulk RNA and then dialyzed, the former against tris-buffer containing Mg^{++} (5×10^{-3} M) and the latter against the same buffer containing Mg^{++} at 5×10^{-5} M. No bulk RNA was added to aliquot C which was dialyzed against buffer containing 5×10^{-3} M Mg^{++}. The dialysis was continued for 24 hours with several changes to provide adequate time for attachment and replacement of any unlabeled complementary RNA, should such exist. At the conclusion of the dialysis, the three preparations were again subjected to centrifugation in linear sucrose gradients each of which contained Mg^{++} at the concentration maintained during the dialysis. The results are described in Fig. 7.

It should be recalled that the profiles of Figs. 6 and 7A were obtained under identical conditions of centrifugation and that the 8S- to 12S-labeled RNA was incubated for 24 hours at high Mg^{++} with the ribosomal components prior to being layered on the sucrose gradient. Comparison of the two provides clear evi-

dence that the pattern observed with samples containing all the original complementary RNA components (Fig. 6) cannot be duplicated with the 8 to 12S variety (Fig. 7A). Indeed, the latter figure bears a striking resemblance to the profiles routinely observed in the investigations (2, 3, 6-8) which isolated informational or messenger RNA by procedures which did not minimize enzymatic degradation.

It will be noted that Fig. 7C contains no optical density profile since no bulk RNA was added to this fraction and the portion of complementary RNA chosen (8 to 12S, Fig. 6) was deliberately selected for the virtual absence of optically observable material. Comparison of the radioactivity profiles in Figs. 7A and 7C allows one to estimate the degree of distortion imposed by the presence of ribosomal RNA on the observed sedimentation of complementary RNA. If attachment of small complementary RNA molecules to ribosomal RNA is to explain the sort

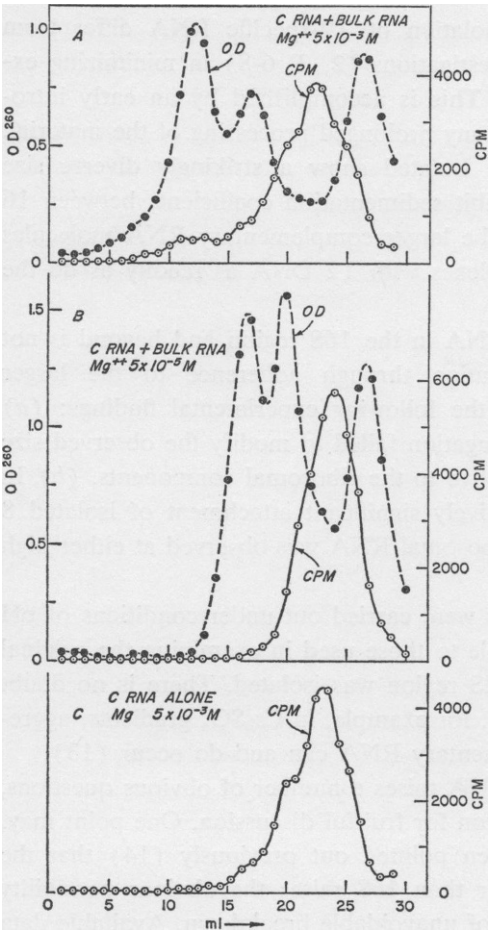

FIGURE 7 *Swinging-Bucket Analysis of 8-12S Complementary RNA.*
The indicated fractions of Fig. 6 were pooled and incubated for 24 hours with the following: (A) unlabeled bulk RNA and 5×10^{-3} M Mg^{++}, (B) unlabeled bulk RNA and 5×10^{-5} M Mg^{++}, and (C) 5×10^{-3} M Mg^{++}. In all cases, the same Mg^{++} levels were maintained in the sucrose gradients.

of radioactivity profiles observed in Figs. 1 and 6, a similar situation should obtain in 7A. We see, however, that the profiles of 7A and 7C are remarkably similar to each other and completely different from those obtained in an analysis of total complementary RNA (Figs. 1 and 6). There is a slight suggestion of association of counts with the 23S region in Fig. 7A, but it involves less than 5 per cent of the radioactive material in the mixture. Similarly, comparing Figs. 7A and 7B reveals no effect of extensive contact with 0.005 M Mg^{++} drastic enough to account for the profiles of Figs. 1 and 6 in terms of attachment of small to large RNA pieces. Finally, it will be noted that there is no hint in the experiments of Fig. 7 of the existence of complementary RNA components larger than 23S, an observation which has been invariable on examination of total complementary RNA samples.

DISCUSSION

The methods employed here for the isolation of T2-specific RNA differ from those used in a number of previous investigations (2, 3, 6-8) in minimizing exposure to enzymatically active extracts. This is accomplished by an early introduction of detergent and phenol prior to any prolonged processing of the material. The labeled RNA molecules ultimately isolated show a strikingly diverse size range in which at least 50 per cent exhibit sedimentation coefficients between 16 and 25S. Hybridizing experiments with the larger complementary RNA molecules established that they form specific complexes with T2 DNA as readily as do the smaller molecules.

That the existence of informational RNA in the 16S region and beyond is not a consequence of fortuitous contamination through adherence to the larger ribosomal components is supported by the following experimental findings: (*a*) Eliminating the Mg^{++} during the centrifugation failed to modify the observed size distribution of complementary RNA relative to the ribosomal components. (*b*) In reconstruction experiments, no quantitatively significant attachment of isolated 8 to 12S complementary RNA to added ribosomal RNA was observed at either high or low Mg^{++}.

It must be emphasized that these tests were carried out under conditions of pH and ionic strength which were comparable to those used in examining the original size distribution from which the 8 to 12S region was isolated. There is no doubt that at high ionic strengths, which obtain, for example, in Cs_2SO_4 gradients, aggregates containing ribosomal and complementary RNA can and do occur (13).

The diverse sizes of complementary RNA raises a number of obvious questions, many of which require further investigation for fruitful discussion. One point may, however, be explicitly noted. It has been pointed out previously (14) that the existence of complementary RNA larger than 16S raises the obvious possibility that the smaller size ranges are artifacts of unavoidable breakdown. Available data

do not permit a decision. It may well be that degraded complementary RNA serves ultimately as precursor for ribosomal RNA as suggested by Kitazume, Yčas, and Vincent (15). Indeed, this is consistent with the findings of Hayashi and Spiegelman (9) who showed with a "chase" experiment that most of the labeled nucleotides of complementary RNA do indeed end up in the stable ribosomal components. But these same experiments indicate that an extensive degradation and reshuffling must intervene since the base composition of the ribosomal RNA, determined by the distribution of radioactivity amongst the four nucleotides, is indistinguishable from the normal and completely different from the base ratio of complementary RNA. This means that very little complementary RNA enters ribosomal RNA as intact polynucleotide sequences.

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