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## THE SELECTIVE SYNTHESIS OF INFORMATIONAL RNA IN BACTERIA\*

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*Introduction.*—For the past several years, this laboratory has employed virus-infected cells to examine the nature of the mechanism involved in transcribing information from DNA for the protein-synthesizing apparatus. The data obtained are consistent with the simplest of transcription devices, namely, that RNA strands complementary to homologous DNA are the informed intermediaries. The experimental steps leading to this conclusion may be briefly noted, since they served as an operational guide in the present attempts to extend this mechanism to uninfected cells.

*Existence proof of RNA homologous to DNA:* The existence of a "T2-specific RNA" inferred from the P<sup>32</sup> experiments of Volkin and Astrachan<sup>1</sup> was established by Nomura, Hall, and Spiegelman.<sup>2</sup> The proof was attained by separating the newly synthesized RNA from the bulk of the cellular RNA using both zone electrophoresis in starch columns and centrifugation in sucrose gradients. The T2-specific RNA was found to have a higher electrophoretic mobility and a greater heterogeneity in size than the three principal normal RNA components (23S, 16S, and 4S). It was further shown that T2-specific RNA was ribosome-bound but with a linkage very sensitive to disruption by low magnesium levels.

*Sequence complementarity:* Having established T2-specific RNA as a physical entity and provided methods for its selective enrichment, it was possible to proceed to an inquiry into the significance of the homology in base ratios between it and T2-DNA. To examine this question, Hall and Spiegelman<sup>3</sup> employed the device described by Marmur and Lane<sup>4</sup> and Doty *et al.*<sup>5</sup> for the reconstitution of double-stranded structures. It was possible to show that RNA-DNA complexes were indeed formed in mixtures of single-stranded T2-DNA and purified T2-RNA subjected to a slow cooling process. The success of the hybridizing experiment suggested immediately that the original observation<sup>1</sup> of a similarity in base composition between T2-RNA and DNA was indeed a reflection of a more profound homology. The fact that hybrid formation was found to be unique to the homologous pair led to the conclusion that the nucleotide sequences of T2-RNA and DNA are complementary.

*Existence of natural RNA-DNA complexes:* If continued formation of complementary RNA is a necessary concomitant, it should be possible to find RNA-DNA hybrids in any cell actively engaged in protein synthesis. Again, the T2-*E. coli*

complex was chosen. By the use of suitable labeling and detecting devices, Spiegelman, Hall, and Storck<sup>6</sup> showed that natural RNA-DNA complexes do indeed exist.

The experiments with the T2-*E. coli* system accomplished two things. They lend evident support for the supposition that information transport from DNA is accomplished via a complementary RNA strand. They provide an operational definition of what we mean by informational RNA. From both the theoretical and empirical viewpoints, the *necessary* and *sufficient* criterion is complementarity of base sequence with homologous DNA as revealed by the hybridization test.<sup>3</sup> Other features of T2-RNA may or may not be shared by other informational RNAs.

We come now to the question of universality, which is the primary concern of the present paper. Can the observations and conclusions derived from the study of the T2-*E. coli* system be generalized to non-infected cells?

The detection and study of the properties of the informational RNA formed in T2-infected cells was greatly facilitated by the fact that the synthesis of the ribosomal RNA components is suppressed. This advantage is not generally present in uninfected cells, which, consequently, complicates the search for normal informational RNA. That it is, nevertheless, feasible is indicated by some recent experiments. Yčas and Vincent<sup>7</sup> infer the existence in yeast of a metabolically unstable and homologous RNA from P<sup>32</sup> experiments similar to those of Volkin and Astrachan.<sup>1</sup> Astrachan and Fisher<sup>8</sup> preliminarily report they have similar findings with bacteria. Finally, Gros *et al.*<sup>9</sup> confirm an earlier<sup>2</sup> observation that short pulses in *E. coli* lead to the appearance of RNA species in the size ranges which characterize T2-informational RNA. Many of these studies involve very short pulses and the consequent synthesis of rather small amounts of RNA. In no case was a rigorous identification of complementary RNA completed.

It would clearly be of great advantage if a circumstance could be found or devised in normal cells which would be analogous to that which occurs on infection with T2. Essentially, what we are demanding is a condition which suppresses ribosomal RNA synthesis and permits the prolonged formation of the informational variety. The possibility that a situation of this sort might, in fact, be realizable was suggested by recent studies<sup>10-12</sup> on RNA and protein synthesis during passage from fast to slow growth.

Several features emerged from these experiments which encouraged us to look more carefully into such transitions as pertinent to the purposes we had in mind. It has been known for some time that the RNA content of a cell is positively correlated with its growth rate. Since the bulk of the RNA is ribosomal, cells growing at higher rates possess more ribosomes.<sup>13</sup> Consider then the situation when one subjects a culture to a "step-down" transition by transferring cells from a rich to a synthetic medium. The growth rate is decreased by a factor of about 2. More important, at the moment they are introduced into synthetic medium and for some time thereafter, the cells have more ribosomes than they can usefully employ. From the viewpoint of selective advantage, it is perhaps not surprising to find<sup>10-12</sup> that such step-down transitions are accompanied by a dramatic cessation of net RNA synthesis. Nevertheless, protein synthesis proceeds for a while at near normal rates. In their relative rates of net protein and RNA synthesis, such cultures are completely analogous to T2-infected cells. It seemed probable that the remaining residue of RNA synthesis would be restricted to the variety immediately

necessary for the fabrication of new protein molecules, i.e., the normal informational RNA for which we were searching. Experiments were, therefore, undertaken to see whether these expectations were realizable.

It is the purpose of the present paper to describe the results obtained. The data show that informational RNA is indeed preferentially synthesized for considerable periods of time in such step-down transitions. The RNA, thus formed, is analogous in all tested characteristics to the T2-specific RNA studied in previous investigations.<sup>1-3, 6</sup>

*Methods and Materials.*—*Bacterial strains:* The bacteria chosen covered the available range of DNA base composition. The following strains were used: *E. coli*, C-122 and B; *Ps. aeruginosa*, ATCC-10197; *B. megaterium*, KM.

*Media buffer and conditions for growth:* The conditions for preparing cells for an experiment are similar to those described previously.<sup>6</sup> The minimal medium (SC) used for *E. coli* was medium C of Roberts *et al.*<sup>14</sup> modified by lowering the phosphate concentration to  $10^{-3}$  M and including 0.1 M tris (hydroxymethyl) aminomethane (Tris) buffered at pH 7.3. *Pseudomonas aeruginosa* and *B. megaterium* were grown in a modified SC medium (FC) in which ferric chloride was replaced with ferric citrate at  $3 \times 10^{-4}$  M and l-glutamate was added to a level of  $3.5 \times 10^{-2}$  M. The enriched medium was a modified<sup>2</sup> Penassay medium (MPM).

The general buffer used, designated by TM, is Tris at 0.01 M and 0.005 Mg<sup>++</sup> buffered at 7.4.

*Radioactive pulses with "step-down" and control cultures:* To obtain "step-down" cultures, logarithmically growing cells in MPM were collected and washed with minimal medium twice. The washed cells were then resuspended in minimal medium and aerated at 30°. Control (non-step-down cultures) were obtained by taking log phase cells growing in minimal medium, subjecting them to the same washing procedure, and resuspending them in minimal medium.

P<sup>32</sup> (20–200  $\mu$ C/ml) was added at the indicated times and intervals. When tritium was the label, H<sup>3</sup>-uridine (1,600  $\mu$ C/ $\mu$ M) was used. The duration of the pulse determined the amount of cold uridine added as a carrier. The final level of uridine ranged from 0.5 to 2  $\gamma$ /ml in the various experiments. At the end of the pulse, cells were dumped into equal amounts of cracked ice made by freezing the appropriate minimal medium to  $-70^\circ$ . The cells were centrifuged, collected, and washed with pre-cooled TM buffer. They were then resuspended in the same buffer at a concentration of about  $1 \times 10^{10}$  cells per ml.

*Preparation of extracts and lysates:* To the cells and buffer, lysozyme (200  $\gamma$ /ml) and DNAase (50  $\gamma$ /ml) were added. The mixture was next frozen at  $-70^\circ$  and then thawed by holding it at 37° for one min. The procedure was repeated twice, then duponol (to a final concentration of 0.4%) was added and the lysates were kept at room temperature for five min. The time of detergent treatment of the lysate was extended to 15 min for *B. megaterium*. The lysates were then chilled and the RNA purified by the phenol procedure of Gierer and Schramm.<sup>14a</sup> After removal of the ether, the RNA in the water layer was precipitated with three volumes of cold ethanol, the precipitate redissolved in TM buffer and reprecipitated once more. After a final solution, the RNA was dialyzed against TM for 12 hr in the cold and again reprecipitated with ethanol.

*Ultracentrifugational analysis:* The characterization of size distribution in RNA preparations was made by centrifugation in linear sucrose gradients using swinging bucket rotors as described previously.<sup>2</sup> The sucrose was buffered with TM. The only modification made in the present study was the use of rotor #SW25. The centrifugation was carried out at 25,000 rpm for 12.5 hr with a rotor temperature of 10°C.

The identification and separation of RNA-DNA hybrids was achieved by equilibrium centrifugation in CsCl gradients as detailed in earlier publications.<sup>2, 6</sup> In all cases, at the end of the centrifugation, the tubes were removed, the bottoms pierced with a needle, and the contents of the tubes collected as separate fractions.

*Optical density and radioactivity measurements:* After suitable dilution, the UV absorption at 260 m $\mu$  of each fraction was taken and aliquots removed for precipitation with trichloroacetic acid and the addition of 100  $\gamma$  of salmon sperm DNA as a carrier for filtration. Counting of radioactivity was carried out as described previously<sup>2, 3</sup> using trichloroacetic acid washing on millipore membranes and the Packard Tri-Carb scintillation counter, which permits the counting of either

$P^{32}$  or  $H^3$ .  $P^{32}$  content of fractions eluted from Dowex columns was determined with the Nuclear thin window gas flow counter.

**Base composition analysis of RNA:** To the RNA preparation, sodium hydroxide was added to a final concentration of 0.3 *N* and the mixture incubated for 20 hr at 30°. The resulting 2'-3' nucleotides were separated using a Dowex formate column with a cross linkage of 8% and a column size of 1 × 5 cm. Eluents used were as follows: 0.005 *N* HCOOH to remove traces of nucleosides and free bases, 0.025 *N* HCOOH to elute cytidilic acid, 0.1 *N* HCOOH for adenylic, 0.05 *N* HCOOH + 0.05 *N* HCOONH<sub>4</sub> for uridylic, and 0.1 *N* HCOOH + 0.2 *N* HCOONH<sub>4</sub> for guanylic. Unlabeled RNA (2-3 mg) of known base composition was added to each sample prior to the hydrolysis step to obtain sufficient O.D. in the four nucleotide regions and provide controls on losses of individual nucleotides. The amount of each nucleotide was calculated using known molecular extinction coefficients. The radioactivity of each fraction was determined with the aid of a nuclear gas flow counter. Since the added carrier was of known base composition, the data permit the calculation of base compositions in terms of both the distribution of the counts in the relevant peaks and the specific activities in the peak regions.

**Experimental Results.—Base ratios of RNA synthesized during a step-down transition:** As a first step, the base composition of the RNA synthesized during a step-down transition was examined. The analysis was carried out by the  $P^{32}$ -labeling procedure described under *Methods and Materials*. To make the comparison with the control culture valid, the  $P^{32}$  pulse was not started until the "step-down" culture had begun logarithmic growth. Two periods of labeling were examined, the first lasting for 10 min and the second for 20 min. Table 1 summarizes the

TABLE 1  
BASE RATIOS OF RNA IN CONTROL AND "STEP-DOWN" CULTURES OF *Ps. aeruginosa*

| Culture   | Time after transfer and interval of pulse | Moles per cent |      |      |      | $\frac{G+C}{A+U}$ | Pu/Pyr. |
|-----------|---|----------------|------|------|------|-------------------|---------|
|           |   | C              | A    | U(T) | G    |                   |         |
| Control   | 20-30                                     | 25.9           | 23.5 | 22.4 | 28.2 | 1.18              | 1.07    |
|           | 20-40                                     | 24.3           | 23.2 | 22.5 | 29.9 | 1.18              | 1.13    |
| Step-down | 20-30                                     | 31.2           | 19.5 | 18.0 | 31.3 | 1.68              | 1.03    |
|           | 20-40                                     | 29.1           | 21.0 | 20.2 | 29.7 | 1.43              | 1.03    |
|           | DNA                                       | 32.0           | 18   | 18   | 32   | 1.77              | 1.00    |
|           | Bulk-RNA                                  | 22.3           | 23.1 | 23.6 | 31.0 | 1.14              | 1.21    |

Both cultures were transferred in log phase to synthetic medium. Control came from synthetic, the "step-down" from complete medium. They were subjected to  $P^{32}$  pulses at the times and for the intervals indicated. The RNA was removed, purified, and hydrolyzed with alkali in the presence of added carrier RNA. The nucleotides in the resulting hydrolysate were separated on Dowex columns and counted and their O.D.s at 260  $m\mu$  were determined. The numbers given are derived from the distribution of the counts and isotope dilution calculations. For purposes of comparison, the total RNA base composition determined from UV absorption data are included for each organism along with the homologous DNA base composition.

data obtained for both the control and the step-down cultures of *Ps. aeruginosa*. Comparison of the two sets of data shows clearly that there is indeed a preferential synthesis of RNA homologous to DNA in the step-down culture and that this synthesis proceeds for extended periods of time subsequent to the transition. The control culture during the same period of time synthesizes an RNA which is very similar in its base composition to the pre-existent bulk RNA.

To examine the generality of this phenomenon, experiments were carried out with several organisms of widely differing DNA base composition. Table 2 summarizes experiments with three different organisms in which  $P^{32}$  pulses were carried out at various periods following a transitional transfer from enriched to minimal medium. It will be noted that in each case the RNA synthesized during the transition mimics the homologous DNA in its per cent GC and purine to pyrimidine ratio. Again, the length of time during which this selective synthesis of homologous

TABLE 2  
BASE RATIOS OF RNA IN "STEP-DOWN" CULTURES

| Organism              | Minutes after Transfer | Moles per cent |      |      |      | %GC  | Pu/Pyr |
|-----------------------|------------------------|----------------|------|------|------|------|--------|
|                       |                        | C              | A    | U(T) | G    |      |        |
| <i>E. coli</i>        | 5                      | 24.7           | 24.1 | 23.5 | 27.7 | 52.4 | 1.07   |
|                       | 60                     | 25.2           | 24.1 | 22.1 | 28.6 | 53.8 | 1.12   |
|                       | DNA                    | 26             | 24   | 24   | 26   | 52.0 | 1.00   |
|                       | Bulk-RNA               | 24.3           | 25.0 | 19.7 | 31.0 | 54.3 | 1.27   |
| <i>Ps. aeruginosa</i> | 5                      | 29.0           | 21.3 | 20.2 | 29.5 | 58.5 | 1.03   |
|                       | 60                     | 27.1           | 21.8 | 21.2 | 29.9 | 57.0 | 1.07   |
|                       | DNA                    | 32             | 18   | 18   | 32   | 64   | 1.00   |
|                       | Bulk-RNA               | 22.3           | 23.1 | 23.6 | 31.0 | 53.3 | 1.21   |
| <i>B. megaterium</i>  | 5                      | 19.7           | 27.9 | 29.0 | 23.4 | 43.4 | 1.05   |
|                       | DNA                    | 19             | 31   | 31   | 19   | 38   | 1.00   |
|                       | Bulk-RNA               | 21.9           | 22.4 | 23.6 | 32.0 | 53.9 | 1.19   |

All cultures were transferred in log phase from complete to synthetic medium at 30°C. At times indicated, they were subjected to a 3-min pulse with P<sup>32</sup>. The RNA was removed, purified, and hydrolyzed with alkali in the presence of added carrier RNA. The nucleotides in the resulting hydrolysate were separated on Dowex columns and counted, and their O.D.s at 260 m $\mu$  were determined. The numbers given are derived from the distribution of the counts and isotope dilution calculations. For purposes of comparison, the total RNA base composition determined from UV absorption data is included for each organism along with the homologous DNA base composition.

RNA continues should be noted. In the case of *Pseudomonas aeruginosa*, even 60 min after the transfer a readily detectable fraction of the RNA formed is homologous to its DNA.

*Size distribution of RNA formed during step-down transitions:* The data summarized in Tables 1 and 2 appeared to confirm our expectation that informational RNA is preferentially synthesized. It was of obvious interest to see whether the other properties of complementary RNA revealed by the study of the T2-*E. coli* complex obtained in the present instance as well.

Informational RNA constitutes a quantitatively minor component and is more heterogeneous in size than the three major components.<sup>3, 15, 16</sup> Consequently, the preferential synthesis of the informational variety is readily revealed as discordancies between radioactive and optical density profiles observed when total RNA from pulsed cells are subjected to density gradient centrifugal analyses. Two cultures, control and "step-down," were treated identically after removal from their respective media. Each was washed and transferred into minimal media. Fifteen min after the transfer, they were subjected to a 15-min H<sup>3</sup>-uridine pulse. The RNA was isolated, purified, and centrifuged on linear sucrose gradients. Figure 1 compares the results obtained in the two cases. In each instance, the O.D. profile readily identifies the 23S, 16S, and 4S RNA components uniformly found in the cells. The parallelism between counts and O.D. in the control is excellent in the 23S and 16S regions. Below this region, we see evidence of discrepancies, a situation which is not surprising, since the control culture would also be expected to make informational RNA. Its presence would not be masked in regions which lack the more stable ribosomal variety. In the step-down culture we find discordancies in all the size ranges of the RNA. It is clear from the radioactive profile that the RNA synthesized in the "step-down" culture is extremely heterogeneous, ranging in size all the way from above the 23S region down to 4S.

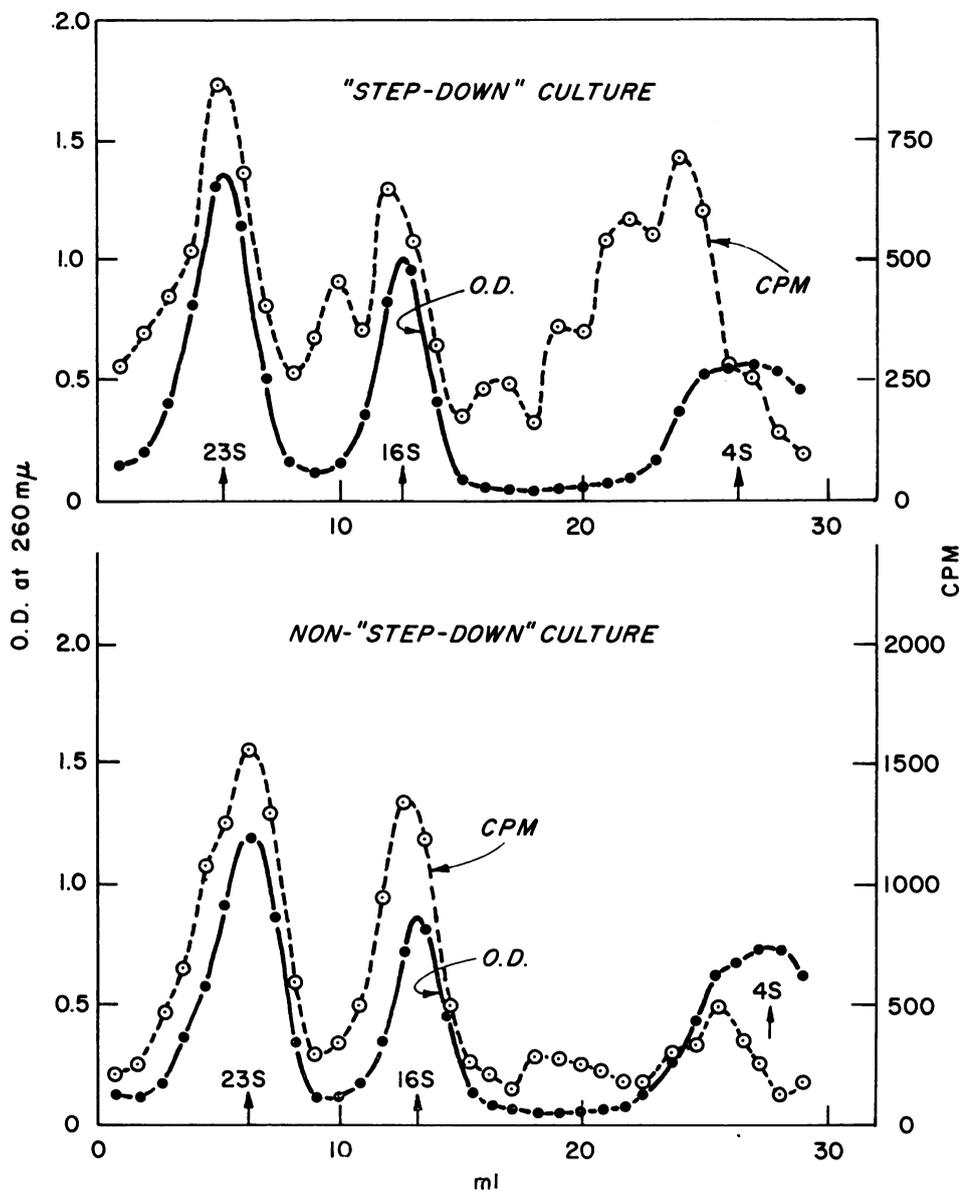
*Ps. Aeruginosa* 15' H<sup>3</sup>-Uridine Pulse; 15 min. after transfer

FIG. 1.—Swinging-bucket analysis in 2.5–15 per cent sucrose gradients of phenol purified RNA. Cells were exposed to a 15-min H<sup>3</sup>-uridine pulse 15 min after transfer into synthetic medium. The upper set comes from cells growing in complete medium before the transfer, the lower from cells in synthetic. In each case, closed circles identify pre-existent and open circles newly synthesized RNA.

This is in agreement with what has been seen in T2-infected and non-infected cells of *E. coli* examined by the procedures employed in the present study.<sup>16</sup>

*Base ratios of the various sizes of RNA synthesized in a step-down culture:* To

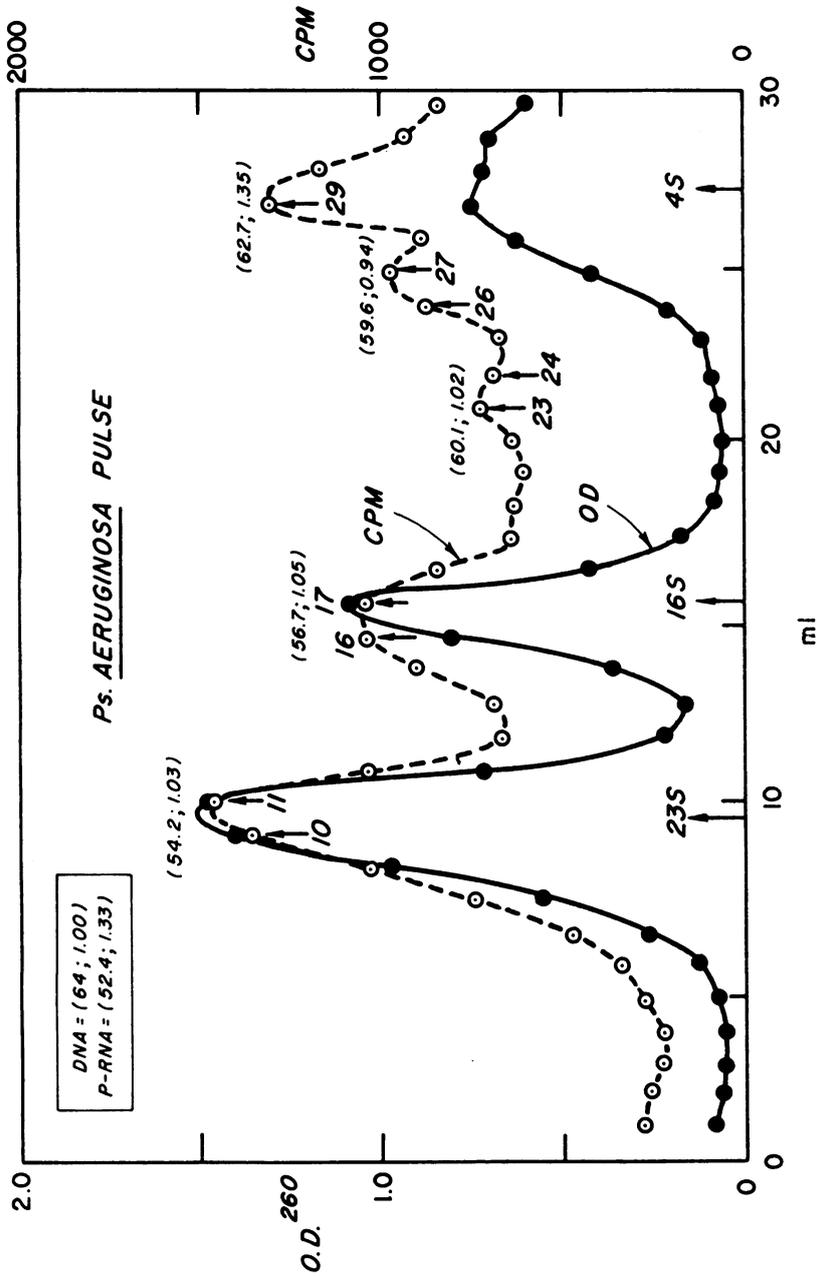


Fig. 2.—Swinging-bucket analysis in 2.5–15 per cent sucrose gradients of phenol purified RNA. Cells were exposed to a 3-min  $P^{32}$  pulse 5 min after they were transferred from complete to synthetic medium. Closed circles identify pre-existent and open circles newly synthesized RNA. The first number in parentheses represents per cent GC and the second the ratio of purines to pyrimidines. Arrows indicate the fractions taken for base composition analysis.

investigate the relation to informational RNA of the various size ranges synthesized during the step-down transition, it was desirable to determine the base compositions of the relevant fractions. In this case, a step-down culture, *Ps. aeruginosa*, was subjected to a 3-min  $P^{32}$  pulse 5 min after log-phase growth had begun, which corresponds to 25 min after the transfer. The ribonucleic acid was purified and examined in a sucrose gradient. The optical density and radioactivity profiles are given in Figure 2. Again, we note a lack of correspondence between the two profiles indicating the preferential synthesis of RNA differing from the three major components.

The fractions indicated by arrows in Figure 2 were analyzed for base composition. For purposes of ready comparison, numbers corresponding to the per cent GC and the purine to pyrimidine ratios characterizing each region are recorded in parentheses. Further details on the base-ratio analyses are given in Table 3.

TABLE 3

*Ps. aeruginosa*: BASE COMPOSITION OF VARIOUS SIZES OF RNA 3-MIN  $P^{32}$ -PULSE OF "STEP-DOWN" CULTURE

| Fraction No.<br>(Fig. 8) | Region | Moles per cent |      |      |      | %GC  | Pu/Pyr |
|--------------------------|--------|----------------|------|------|------|------|--------|
|                          |        | C              | A    | U(T) | G    |      |        |
| 10, 11                   | 23S    | 25.9           | 22.4 | 23.4 | 28.3 | 54.2 | 1.03   |
| 16, 17                   | 16S    | 27.0           | 21.5 | 21.8 | 29.7 | 56.7 | 1.05   |
| 23, 24                   | 10-12S | 30.3           | 20.9 | 19.0 | 29.8 | 60.1 | 1.02   |
| 26, 27                   | 6-8S   | 31.2           | 19.8 | 20.6 | 28.4 | 59.6 | 0.94   |
|                          | DNA    | 32             | 18   | 18   | 32   | 64   | 1.00   |
|                          | P-RNA  | 22.4           | 26.8 | 20.7 | 30.1 | 52.5 | 1.30   |

Conditions of experiment and analyses similar to those described in Table 2. The fractions taken are those indicated by arrows in Figure 2. P-RNA means purified ribosomal RNA, and the base composition was obtained from UV absorption data of the nucleotides.

Comparison of the parameters reveals that DNA-like RNA of all size classes have been synthesized, confirming the findings recorded in the previous sections. As one proceeds to the smaller size ranges (16S to 6S), the homology between the RNA and the DNA becomes excellent.

*Metabolic stability of RNA synthesized in step-down cultures:* One of the features thus far found to be characteristic of complementary RNA is a high turnover rate. This question was examined with respect to the RNA synthesized during a step-down transition. A culture of *Ps. aeruginosa* was subjected to a  $P^{32}$  pulse in exactly the same manner as that detailed in the experiments described by Figure 2 and Table 3. However, twice as much radioactivity was used. Following the 3 min of labeling, the culture was centrifuged, washed, and reintroduced into the same medium containing  $P^{31}$ . It was then allowed to "chase" for 0.7 generations. Figure 3 describes the optical density and radioactivity profiles observed when the purified RNA from this preparation was centrifuged in a sucrose gradient in the usual manner.

Comparison of the radioactive profiles in Figures 2 and 3 provides clear evidence of the metabolic instability of the heterogeneous RNA synthesized during the transition period. The chasing interval in the  $P^{31}$  medium eliminated almost completely the discordancies between the optical density and radioactivity profiles observed in the initial pulse (Fig. 2). To provide further information on this question, the fractions indicated by arrows in Figure 2 were taken for base-composi-

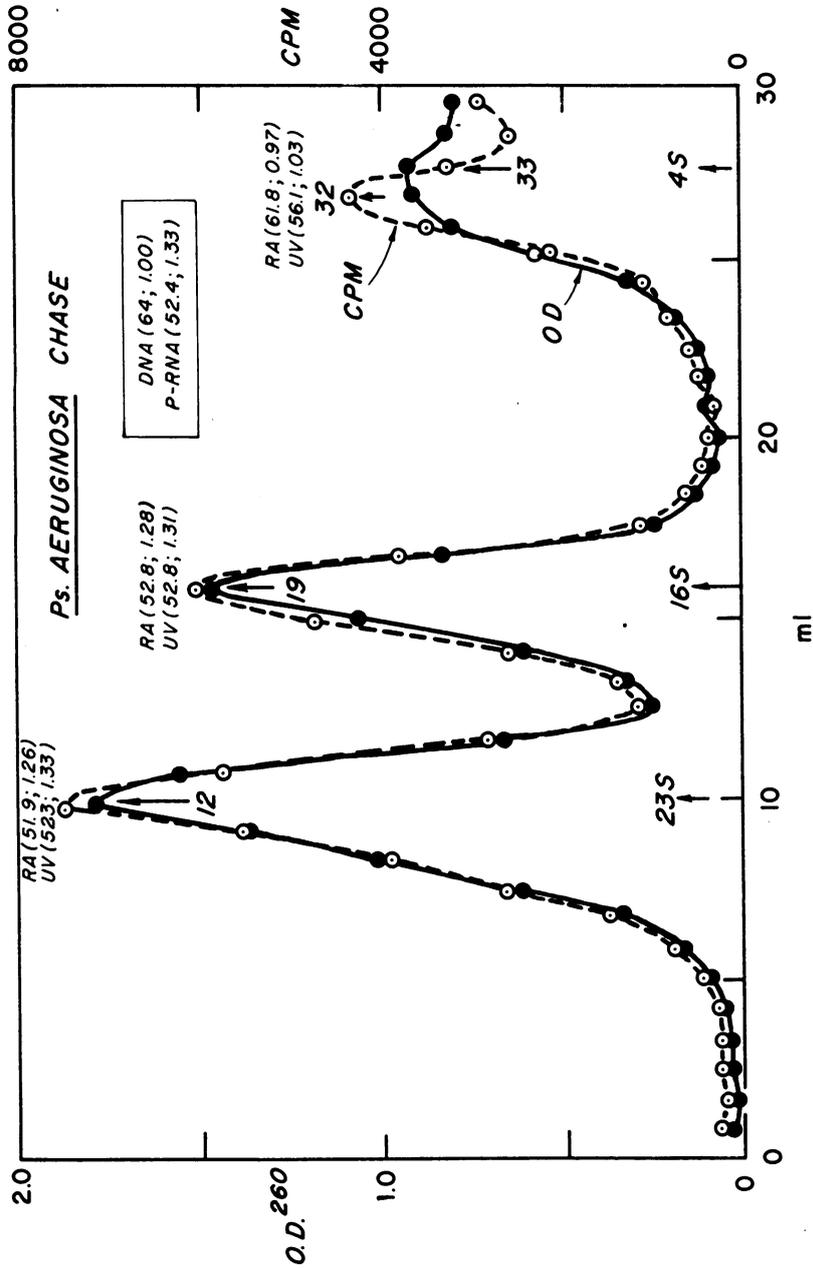


Fig. 3.—Swinging-bucket analysis in a 2.5–15 per cent sucrose gradient of phenol purified RNA. Cells were treated similarly to those used in the experiment of Figure 2. They were then exposed to a “chase” for 0.7 generations in non-radioactive synthetic medium. The first number in parentheses represents per cent GC and the second, ratio of purines to pyrimidines. The values found by both UV absorption and radioactivity are given. Arrows indicate fractions subjected to base composition analysis.

tion determinations. The numbers in parentheses give the results in terms of per cent GC and purine to pyrimidine ratios. Table 4 provides further details on the

TABLE 4  
BASE COMPOSITION OF RNA OF DIFFERENT SIZES SUBSEQUENT TO CHASE OF THE CULTURE OF TABLE 3

| Fr No. | Region | <i>Ps. aeruginosa</i> |      |      |      |      |      |      |      |      |      |        |      |
|--------|--------|-----------------------|------|------|------|------|------|------|------|------|------|--------|------|
|        |        | Mole per cent         |      |      |      |      |      | %GC  |      |      |      |        |      |
|        |        | C                     |      | A    |      | U    |      | G    |      | GC   |      | Pu/Pyr |      |
| CPM    | UV     | CPM                   | UV   | CPM  | UV   | CPM  | UV   | CPM  | UV   | CPM  | UV   |        |      |
| 12     | 23S    | 22.2                  | 22.1 | 26.0 | 26.9 | 22.1 | 20.8 | 29.7 | 30.2 | 51.9 | 52.3 | 1.26   | 1.33 |
| 19     | 16S    | 22.2                  | 22.8 | 25.5 | 26.7 | 21.7 | 20.5 | 30.6 | 30.0 | 52.8 | 52.8 | 1.26   | 1.31 |
| 32, 33 | 4S     | 31.9                  | 29.2 | 19.4 | 24.4 | 18.8 | 20.5 | 29.9 | 25.9 | 61.8 | 56.1 | 0.97   | 1.03 |
|        | DNA    |                       | 32   |      | 18   |      | 18   |      | 32   |      | 64   |        | 1.00 |
|        | P-RNA  |                       | 22.4 |      | 26.8 |      | 20.7 |      | 30.1 |      | 52.5 |        | 1.30 |
|        | S-RNA  |                       | 29.2 |      | 24.4 |      | 20.5 |      | 25.9 |      | 56.1 |        | 0.97 |

A culture treated similarly to the one used in the experiment of Table 3 and Figure 2 was taken after the 3-min  $P^{32}$  pulse, washed, and allowed to grow for 0.7 generations in an unlabeled medium. The RNA was prepared and analyzed in the usual way. The data from the UV absorption are included to permit a comparison of the degree of correspondence between the radioactive and UV calculation on the same samples. The fractions taken are indicated by the arrows in Figure 3. The data obtained on P-RNA and S-RNA were from UV absorption data on separately purified material. P-RNA has the same meaning as in Table 3. S-RNA is the RNA remaining in the supernatant after removal of ribosomes by means of a 38K spin for 6 hr.

base ratios determined by both the ultraviolet absorptions derived from the added carrier and the distribution of radioactive counts among the 2'-3' nucleotides as eluted from a Dowex column.

The cold carrier added to each sample examined consisted of RNA isolated from the region corresponding to the radioactive fraction being analyzed. As is evident from the data detailed in Table 4, the compositions of the labeled RNA in the 23S and 16S regions are now typically ribosomal. The base ratios determined by distribution of radioactive counts and ultraviolet absorption are now in excellent agreement.

It should be noted that despite the fact that the chase extended for a period of 0.7 generations there is still a detectable discrepancy in both the profiles of Figure 3 and the base compositions in the 4S region (Table 4). This may be a reflection of the difficulty of completely removing the informational RNA from the 4S region. These observations would be consistent with a mechanism which involves a comparatively rapid breakdown of the larger informational RNA pieces to 4S size and the slower conversion of these to the level of nucleotide derivatives.

One other point of interest may be briefly mentioned. The total amount of acid-precipitable radioactivity found in the RNA at the end of the chase period corresponded to 150 per cent of that which was incorporated during the 3-min pulse. Consequently, of the total amount of labeled ribosomal RNA synthesized during the chasing period, 30 per cent came from a nucleotide pool and 70 per cent was derived from the informational RNA which had been synthesized during the initial labeling period. Nevertheless, the radioactive base compositions of the 23S and 16S regions are indistinguishable from those normally found for ribosomal RNA. This suggests that the informational RNA does not enter as intact polynucleotide into the ribosomal components.

*Hybridizability of RNA synthesized in step-down transitions:* The experiments described thus far establish that step-down cultures preferentially synthesize a type of RNA which is heterogeneous in size and metabolically unstable and possesses an over-all base composition analogous to its homologous DNA. These are fea-

tures expected of informational RNA on the basis of our previous experience with the T2-system. To complete the identification, it was necessary to test for sequence complementarity by the hybridization procedure. An extensive study<sup>17</sup> was made on this question, which will be detailed elsewhere. We cite here a few representative experiments illustrating the principal features and findings. The general procedures employed may be outlined as follows:

- (1) Step-down cultures were pulsed with tritiated uridine to label the RNA synthesized during transition.
- (2) The RNA was isolated and purified by the phenol method.
- (3) The purified RNA was separated according to size on sucrose gradients.
- (4) Different regions of the radioactive profile were collected and concentrated.
- (5) Hybridizing tests were carried out by exposing mixtures of the labeled RNA and single-stranded DNA to a slow cool from 55° to 28°C in 25 hr.
- (6) The resulting mixtures were then subjected to an equilibrium centrifugation in cesium chloride gradients according to the methods described by Hall and Spiegelman.<sup>3</sup>

Figure 4 shows the outcome of a hybridization carried out between single-stranded *E. coli* DNA and 8-12S tritiated RNA labeled with H<sup>3</sup>-uridine during a step-down transition. It will be noted that excellent hybridization occurs. The shoulder in the optical density profile on the light side corresponds to marker double-stranded DNA. That the interaction is specific is shown in Figure 5, in which a similar hybridizing attempt was made between the same RNA fraction and single-stranded DNA derived from *Pseudomonas aeruginosa*. There is no suggestion of any detectable mating.

Similar experiments were carried out with *Ps. aeruginosa*. The RNA again was labeled with H<sup>3</sup>-uridine during a step-down transition. Figure 6 shows the outcome of a hybridization carried out with homologous single-stranded DNA and H<sup>3</sup>-RNA isolated from the 16S region. Here again, we note excellent hybrid formation as demonstrated by the peak of tritium in the DNA region. This same figure illustrates a feature which is extremely useful in attempts at detecting hybrid and distinguishing it from non-specific aggregation. Aliquots from each of the tubes were taken and treated with 5γ/ml of RNAase for 15 min at room temperature. Carrier DNA was then added and the material reprecipitated, washed, and counted. It will be noted that the radioactivity corresponding to uncombined RNA is almost completely removed by the RNAase treatment. However, the counts in the region of the hybrid are clearly much more resistant to hydrolytic cleavage. It should be noted in passing that hybridized RNA is not completely resistant, since exhaustive treatment with RNAase can result in complete loss of acid-precipitable counts.

Specificity tests with informational RNA from *Ps. aeruginosa* yielded results similar to those described for *E. coli*. No interaction with heterologous single-stranded DNA was observed.

The experiments summarized in the present section establish that RNA molecules preferentially synthesized in step-down cultures possess base sequences complementary to their homologous DNA.

*Discussion.*—It is apparent from the data presented that the choice of cultures in “step-down” transition was a happy one. They obviously provide almost ideal

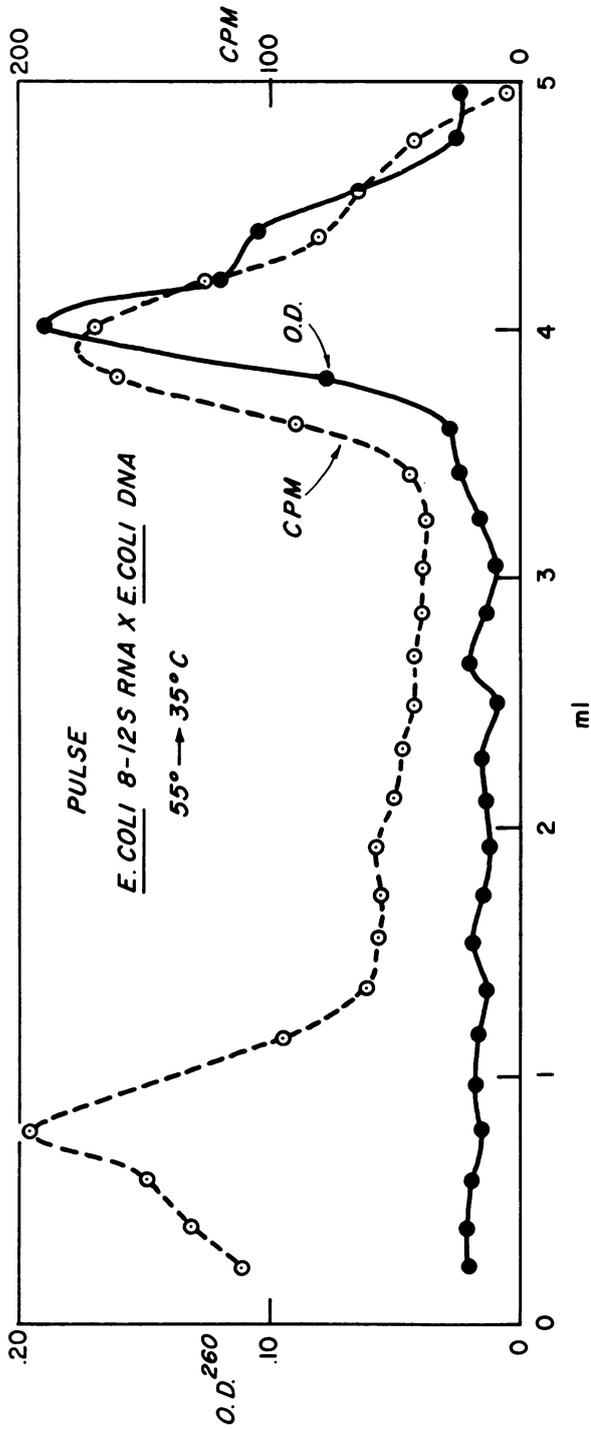


FIG. 4.—Equilibrium density centrifugation (33 K 60 hr) in  $\text{CsCl}$ . A mixture of  $\text{H}^3$ -RNA (8-12S) from an *E. coli* "step-down culture" slow cooled with single-stranded *E. coli* DNA. Double-stranded *E. coli* DNA was added as a marker and is represented by the shoulder on the light (right) side of the main O.D. peak.

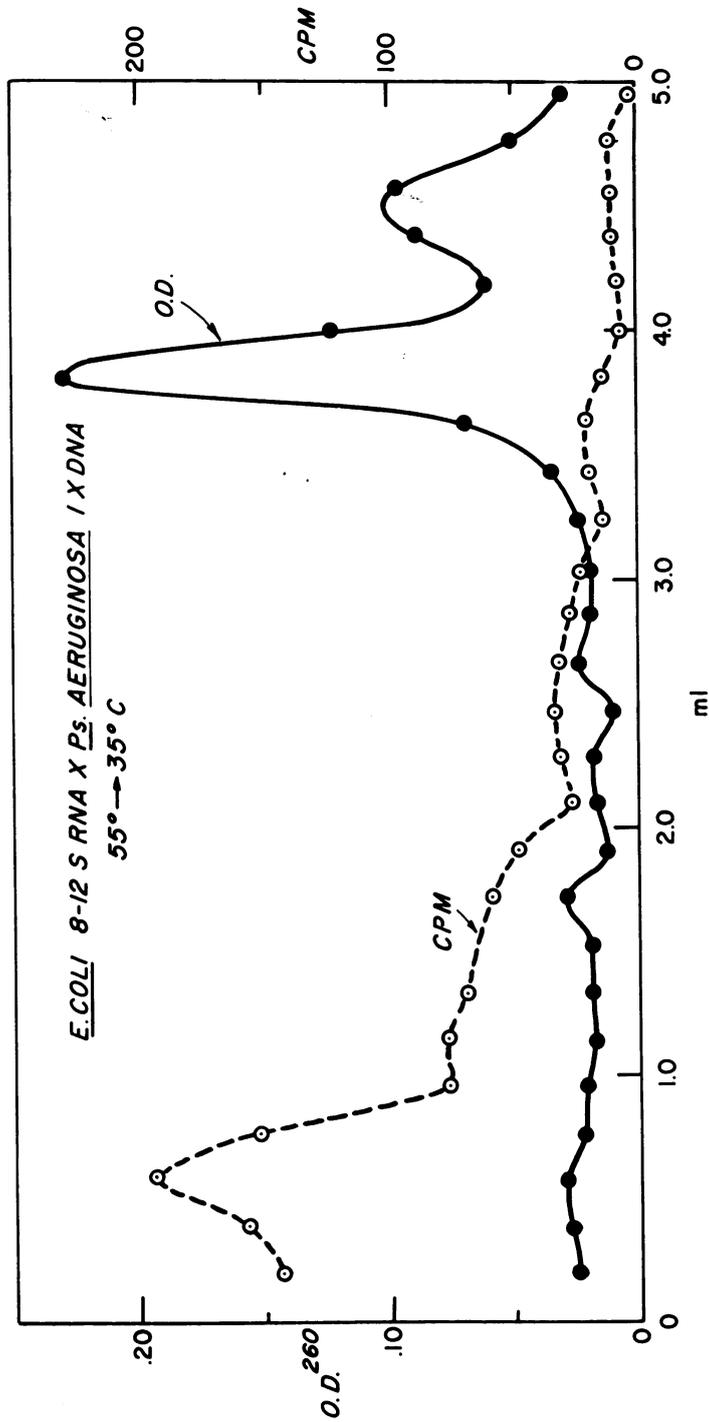


FIG. 5.—Equilibrium density centrifugation (33 K 60 hr) in CsCl. A mixture of H<sup>3</sup>-RNA (8-12S) from an *E. coli* "step-down" culture slow cooled with single-stranded DNA from *Ps. aeruginosa*. Double-stranded *E. coli* DNA was added as a marker and is represented by the second peak to the right.

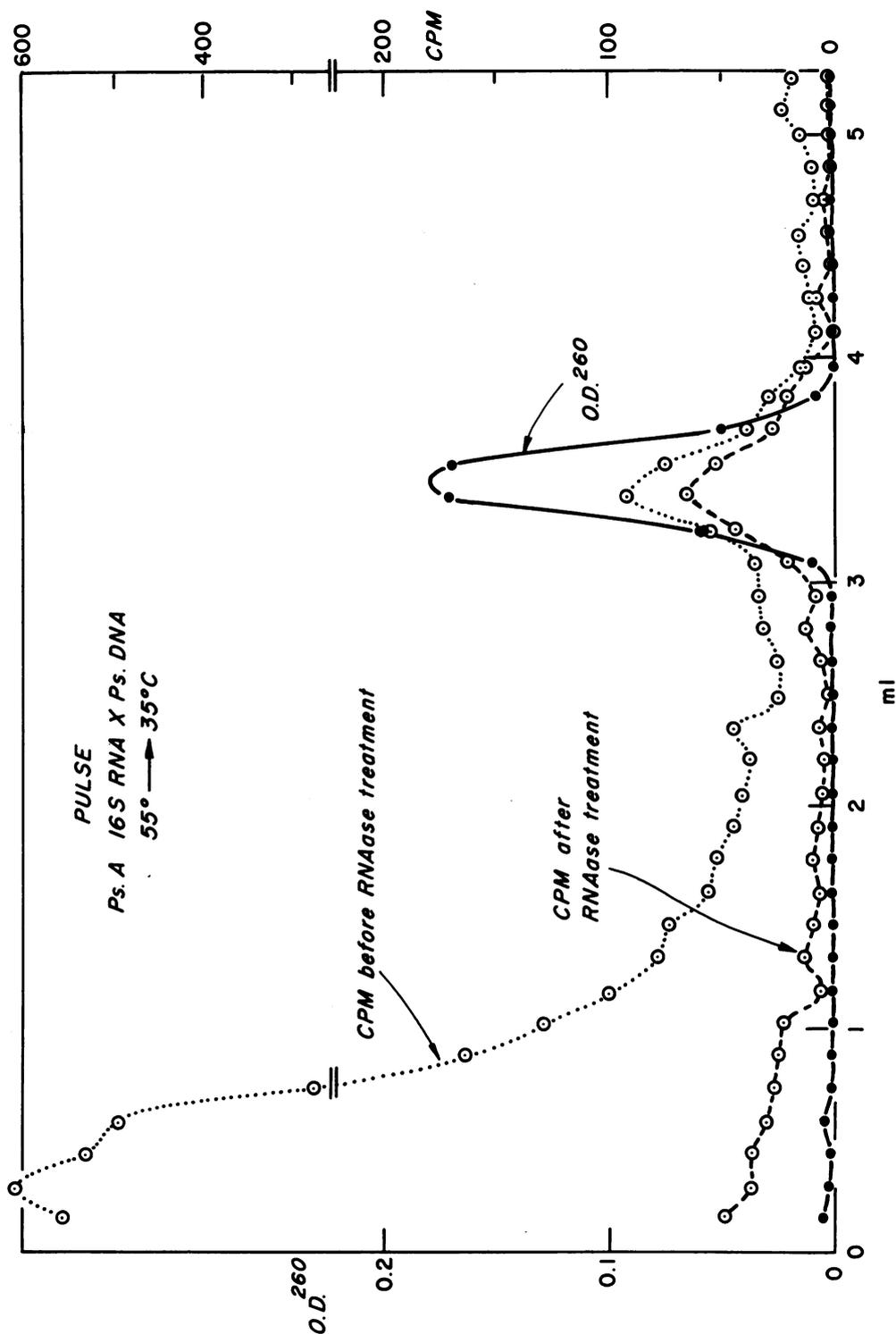


FIG. 6.—Equilibrium density centrifugation (33 K 60 hr) in CsCl. A mixture of H<sup>3</sup>-RNA (16S) from a *Ps. aeruginosa* “step-down” culture slow cooled with single-stranded DNA from *Ps. aeruginosa*. No marker was added. Open circles-dashed line gives the effects of treatment of the indicated fractions with R.N.Aase prior to precipitation and counting.

experimental material for the study of non-ribosomal RNA synthesis. In particular, they permitted the ready demonstration of RNA molecules in non-infected cells which satisfy the complementarity criterion established with the T2-*E. coli* complex.

Noteworthy is the fact that other features of T2-complementary RNA are shared by the bacterial informational RNA revealed by the present investigation. These include homology of base composition with the relevant DNA, metabolic instability and heterogeneity in size. Caution should, however, be exercised in accepting any of these as diagnostic of an RNA complementary to a specific DNA. None of them, including homology of base composition, need universally characterize or be unique to informational RNA. A few examples may be cited. T2-RNA is homologous in base ratio but not complementary to T5-DNA.<sup>3</sup> Conversely, a complementary RNA might exhibit non-homologous base ratios if it represented a copy of sufficiently small DNA segment. Further, one need only recall hemoglobin synthesis in the reticulocyte to entertain some doubts concerning the universality of metabolic instability for all informational RNA. Obviously, neither size range nor magnesium-dependent adsorbability to ribosomes can be accepted as uniquely identifying characteristics of RNA types. The point being emphasized is that none of these secondary properties can either alone, or in combination, be accepted as substitutes for complementarity as the criterion for informational RNA. Finding an RNA with one or more of these features suggests, but does not establish, that a complementary RNA has been identified.

Along similar lines, a few clarifying comments on terminology may be made. The terms "complementary" and "informational" have been used interchangeably both in the present and previous discussions<sup>3, 6, 15</sup> of the RNA molecules with which we are concerned. Every complementary RNA is informational in at least one sense. Even if it is a complementary copy of a nonsense sequence, it nevertheless contains the information necessary to specify the base order of its parental DNA.

It is evident from the experiments described that these terms have well defined operational definitions. A given RNA molecule is defined as falling within the informational class if its sequence is complementary to a specific DNA molecule. At present, the most sensitive available test for sequence complementarity is the hybridization experiment of Hall and Spiegelman.<sup>3</sup>

It is important to emphasize that the word "informational" is not suggested as a substitute for the term "messenger" introduced in the elegant theorizations of Jacob and Monod.<sup>18</sup> It seems likely that both terms will be useful. Thus, a given messenger RNA is presumed to constitute the structural program for the synthesis of a particular protein. It obviously must, therefore, be informational. However, not all informational RNA need operate as messengers. It is conceivable that complementary RNA molecules will be found which serve regulatory rather than programming functions. At the present time, there exists no operational definition of "messenger" RNA. This will presumably emerge from the *in vitro* systems being developed by Nisman *et al.*<sup>19</sup> and Novelli<sup>20</sup> and his collaborators.

Over the past several years, considerable progress has been recorded on the enzymological aspects of DNA-dependent RNA synthesis.<sup>21-27</sup> It seems likely that we will soon be in possession of the enzymatic details operating in the syn-

thesis of complementary RNA.

It is comforting that a number of laboratories, using experimental approaches similar<sup>19</sup> to and different<sup>28</sup> from ours, are arriving at equivalent views of the genetic transcription mechanism. The pleasant air of agreement thus generated should not lull us into forgetting that no one has as yet proved that complementary RNA molecules perform the functions we hope they do.

*Summary.*—The primary purpose of the present paper was to determine whether normal cells synthesize the type of informational RNA which had been detected in T2-infected cells. The defining feature of this RNA is that it be capable of forming hybrids with homologous single-stranded DNA. For reasons which are detailed in the text, it was suspected that cells subjected to a shift from a rich to a synthetic medium would preferentially synthesize such complementary RNA. This expectation was fully realized. Use of such “step-down” cultures facilitated the ready exhibition of an RNA in uninfected cells having all the properties which had been established for the T2-complementary RNA. This normal informational RNA exhibits a base ratio analogous to its homologous DNA, is metabolically unstable and very heterogeneous in size, and possesses the ability to hybridize specifically with its homologous DNA.

It would appear from the results summarized here that the synthesis of polyribonucleotide strands complementary to homologous DNA is a generalized feature of normal and virus-infected bacterial cells. With respect to the genetic transcription mechanism, the present data are consistent with the conclusions drawn from our previous experiments with T2-infected cells. It would appear that complementary RNA strands are the intermediaries between DNA and the protein-synthesizing apparatus.

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## CHARACTERISTICS AND STABILIZATION OF DNAASE-SENSITIVE PROTEIN SYNTHESIS IN *E. COLI* EXTRACTS

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It has been assumed for many years that in protein synthesis the base sequence of DNA specifies the base sequence of RNA and that RNA in turn controls the amino acid sequence of protein. In accord with this notion, several groups recently have observed an inhibition of amino acid incorporation into protein by DNAase in cell-free extracts.<sup>1-3</sup> One object of the present investigation was to study this phenomenon further.

A major difficulty in the study of cell-free protein synthesis in *E. coli* systems has been the necessity for preparing fresh enzyme extracts for each experiment. Techniques have not been available for stabilization and storage of enzyme extracts comparable to the techniques available for mammalian systems.<sup>4</sup> In the present communication, an amino acid-incorporating system stable to storage for several months will be described. The characteristics of amino acid incorporation into protein by the stored extracts were investigated also. A part of these data has been presented in a preliminary report.<sup>2</sup>

*Methods and Materials.*—*E. coli* W3100 cells, harvested in early log phase, were washed by centrifugation and disrupted by grinding with twice their wet weight of alumina A301 (Aluminum Corporation of America) for 5 min at 5°. All subsequent steps were performed at this temperature. The enzymes were extracted with buffer containing 0.01 M Tris(hydroxymethyl)amino-methane, pH 7.8; 0.01 M magnesium acetate; 0.06 M KCl; and 0.006 M mercaptoethanol (standard buffer) equivalent to two or three times the wet weight of cells. The extract was centrifuged three times at 30,000 × g for 20, 20, and 60 minutes, respectively. The pellets were discarded after each centrifugation. The final supernatant fluid (S-30) was centrifuged at 105,000 × g for 2 hr in the Spinco Model L ultracentrifuge to sediment the ribosomes. The supernatant solution (S-100) was aspirated, and the ribosomes were suspended in standard buffer by gentle homogenization in a Potter-Elvehjem homogenizer and were washed by centrifuging again at 105,000 × g for 2 hr. The supernatant fluid was decanted and discarded, and the ribosomes (W-Rib) were suspended in the original volume of standard buffer. Fractions S-30, S-100, and W-Rib were dialyzed against 60 volumes of standard buffer overnight at 5° and were stored in small aliquots at -15° until needed.

DNAase I, RNAase, and trypsin were crystalline preparations obtained from the Worthington