

The Relation of Informational RNA to DNA

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I. INTRODUCTION: NATURE OF THE PROBLEM

The essence of the dogma, so acceptable to our time, can be briefly summarized by the following familiar diagram:

DNA → RNA → Protein.

Here, the arrows are meant to indicate flow of information. The presumption is that the genetic information coded in the base sequence of DNA is ultimately transcribed into the amino acid sequence of protein via a polyribonucleotide intermediary. It is the primary purpose of the present paper to focus attention on the first step in this chain of events.

The simplest imaginable transcription mechanism one can propose would suggest the synthesis of complementary RNA copies of the DNA. The RNA so formed would mimic the base ratio of its parental DNA providing one of the two following conditions was satisfied.

(a) Both strands of the DNA are employed as templates for complementary RNA synthesis, or

(b) The over-all base composition of the two complementary strands of DNA are the same or nearly so.

Comparison of the base compositions of the total RNA among bacteria of widely differing DNA compositions does not encourage the belief that a major proportion of the cellular RNA is analogous to its homologous DNA. Examination of the principal RNA molecular species reveals a surprisingly uniform picture among rather unrelated organisms. Three major components are universally found which are distinguishable by their sedimentation constants (23S, 16S, and 4S). The 23S and 16S varieties have the same base compositions, are found in the ribosomes (Kurland, 1960), and constitute approximately 85% of the total RNA of the cell. The 4S variety has, in general, a different base ratio and is found in the supernate or "soluble" fraction.

Thus far, none of the three major components have exhibited any detectable correlation in their base ratio with the DNA composition of the cells from which they are derived. This is illustrated in Fig. 1 with respect to ribosomal RNA. Figure 1 reproduces Belozersky and Spirin's (1958) relative plot of total RNA base composition (open circles) versus homologous DNA and includes as solid circles data on ribosomal RNA (23S + 16S) obtained from *D. pneumoniae*, and

M. lysodeikticus (Woese, 1961), *E. coli*, *Ps. aeruginosa*, and *B. megaterium* (Hayashi and Spiegelman, 1961). Although the information on ribosomal RNA is still scanty, the five samples available do derive from organisms which extend over a considerable portion of the GC range of DNA base composition. Nevertheless, the ribosomal (G + C/A + U) ratios fall within rather narrow limits and show no correlative tendency. Such data suggest that the comparatively slight correlation established by Belozersky and his co-workers between bulk RNA and homologous DNA is to be ascribed to a small fraction of the cellular RNA. If, therefore, hope is to be retained for the existence of the simple transcription mechanism proposed above, it will have to be assumed that a quantitatively minor component of the RNA is involved in transferring information from DNA to the protein synthesizing machine. From the viewpoint of such considerations, the normal cell would not appear to represent ideal material for the initial search for the RNA which programs protein synthesis.

Several years ago my colleagues and I decided to turn our attention to the virus infected cell. The bacterial-virus complex possessed a number of obvious attractive advantages for the experimental analysis of the relations among DNA, RNA, and protein. In the first place, the data accumulated over the past 15 years suggested that infection of bacterial cells with virulent viruses is followed by a restriction of protein and nucleic acid synthesis to a comparatively small class of macromolecules relevant to the formation of viral components. Second, and more important, were the experiments of Volkin and Astrachan (1956) who demonstrated the incorporation of P^{32} into an RNA fraction which was distinguishable by its apparent base ratios from the bulk of the host's RNA. These ratios were determined by estimation of the relative P^{32} content of the corresponding 2'-3' nucleotides isolated from an alkaline hydrolysate of RNA from infected cells. Of greatest interest was the fact that the numbers so obtained appeared to mimic the DNA base ratio of the infecting particle. Here, we have the first suggestion of a complementary RNA which could play an informational role in the synthesis of a particular class of proteins. The intriguing implications of these observations made it mandatory that they be extended in several directions.

The existence of an RNA peculiar to virus infected

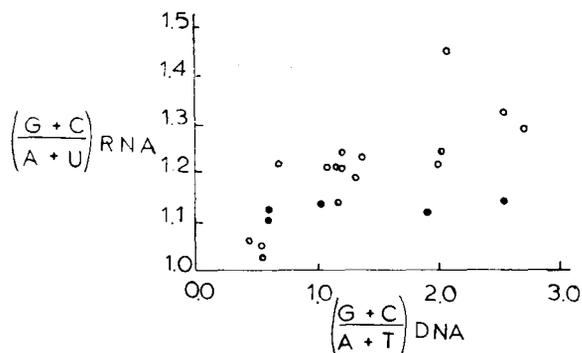


FIGURE 1. Relative plot of base composition of RNA and DNA from the same organisms. Open circles are Belozersky's (1959) data on total RNA from 22 bacteria. Closed circles are data on ribosomal RNA from five bacteria (Woese, 1961; Hayashi and Spiegelman, 1961).

cells is inferred from the Volkin-Astrachan experiments solely on the basis of the distribution of isotope among the four nucleotides. In the absence of further information, it was possible to interpret these results in terms of unequal pool labeling and other conceivable complications. It was clearly of importance to provide independent evidence in support of their inference. The unequivocal proof of the existence of a "T2-specific" RNA would immediately generate a host of new experimental possibilities. As a convenient guide to the following discussion, we may set down the questions which stimulated us to the performance of the experiments we will describe.

1) Is it possible to effect a physical separation of the RNA formed subsequent to T2-infection and thus provide proof that "T2-specific RNA" exists?

2) What relation, physical or other, does the T2-specific RNA have to the normal RNA containing components of the cell?

3) Is the similarity of base ratios between T2-RNA and DNA a reflection of a more detailed identity in terms of base sequences? More specifically, are they complementary?

4) Can one find evidence of naturally occurring RNA-DNA complexes predicted by the assumption that RNA complementary copies are the normal intermediates in the flow in information from the genome?

5) Does a similar type of complementary RNA also exist in normal non-infected cells?

It will be noted that the posing of each question makes sense only if the preceding one has been answered in the affirmative. We now undertake to describe the nature of the experiments which led us to these conclusions.

II. PHYSICAL PROPERTIES AND LOCATION OF T2-SPECIFIC RNA

Nomura, Hall, and Spiegelman (1960) undertook to obtain information pertinent to the first two

questions raised. In addition to radioactive labeling, two techniques were used in this investigation to examine the nature of the RNA synthesized following infection with T2. One involved zone electrophoresis through starch columns (Rotman and Spiegelman, 1954). The other employed centrifugation through linear sucrose gradients developed by Britten and Roberts (1960). These procedures were used to examine isolated ribosomes and purified RNA prepared from ribosomes by the phenol procedure of Gierer and Schramm (1956). The data so obtained should detect differences in size, electrophoretic mobility, and degree of association with ribosomes.

An examination was first made of the distribution of RNA synthesized subsequent to T2-infection. Here, care was exercised to use cell breakage and extraction conditions which insured integrity of the ribosomes. It was found that with such methods most (63%) of the newly synthesized RNA was ribosome bound. However, the association of the new RNA involved a linkage which was much more labile to low Mg concentrations than that which characterizes normal ribosomal RNA.

This is illustrated in Fig. 2 which describes the results of electrophoresis experiments of ribosome fractions obtained from T2-infected cells. In these experiments, free ribosomal RNA was included as a marker and is represented by the leading peak of the optical density profile. It will be noted that at 5×10^{-3} M Mg^{++} the bulk of the newly synthesized RNA (P^{32} labeled) travels with the ribosomes although there is some apparent dissociation evident even at this level of magnesium. When the magnesium concentration is lowered to 1×10^{-4} M most of the radioactive RNA leaves the ribosomes and travels as free RNA. Under similar conditions, no detectable separation of normal ribosomal RNA from the nucleoprotein particles can be detected.

Most significantly, it will be noted from the lower half of Fig. 2 that the liberated labeled RNA moves faster than the added carrier *E. coli* RNA. This difference in electrophoretic mobility provided the first evidence that the RNA synthesized in a T2-infected cell was indeed a distinct entity which could be physically separated from the bulk of the pre-existent ribosomal RNA. This property was examined in greater detail with purified RNA isolated by the phenol method. Figure 3 compares the results of an electrophoretic analysis of ribosomal RNA prepared from T2-infected and control cells subjected to P^{32} pulses of the same duration. It is clear that virtually all of the RNA synthesized in the T2-infected cell possesses a higher electrophoretic mobility. In the case of the non-infected cell, the major proportion of the RNA synthesized possesses a mobility very similar to that of the non-labeled pre-existent ribosomal RNA.

Another difference between normal and infected

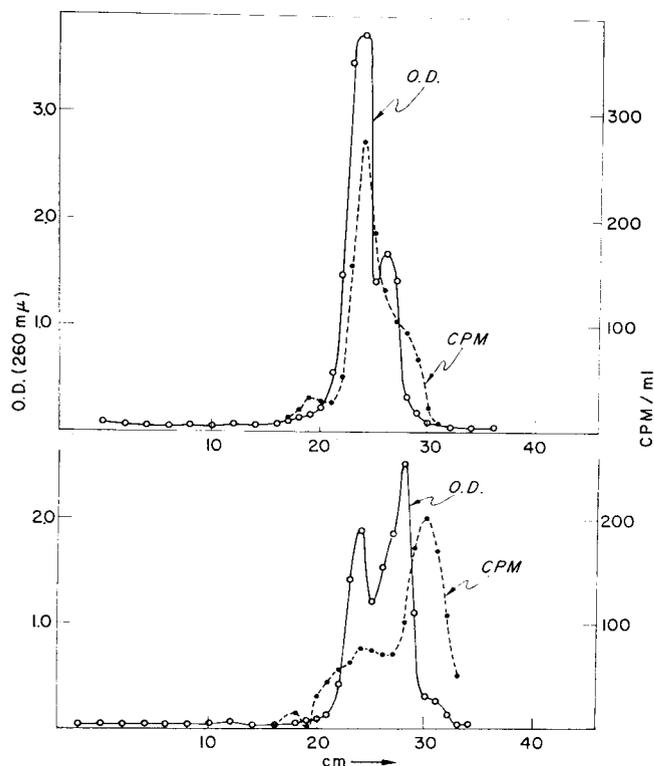


FIGURE 2. Starch column electrophoresis of the ribosome fraction of T2-infected cells. (a) Electrophoresis in 0.03 M-tris, pH 7.8 with 5×10^{-3} M Mg^{++} after 3 hr dialysis against this solvent. Duration of run—13 hr at 8.0 v/cm; 11 ma. (b) Electrophoresis in 0.03 M-tris, pH 7.8, 1×10^{-4} M Mg^{++} after 24 hr dialysis against this solvent. Duration of run—13 hr at 6.0 v/cm; 4 ma. Ribosomes used in this experiment came from T2-infected cells exposed to P^{32} between 5 and 7 min after infection. *E. coli* particle RNA (prepared by the phenol method) was added as a marker just before electrophoresis. After elution of each fraction from starch OD at 260 $m\mu$ was measured; then the acid insoluble P^{32} which was solubilized by RNAase was counted (counts/min curve).

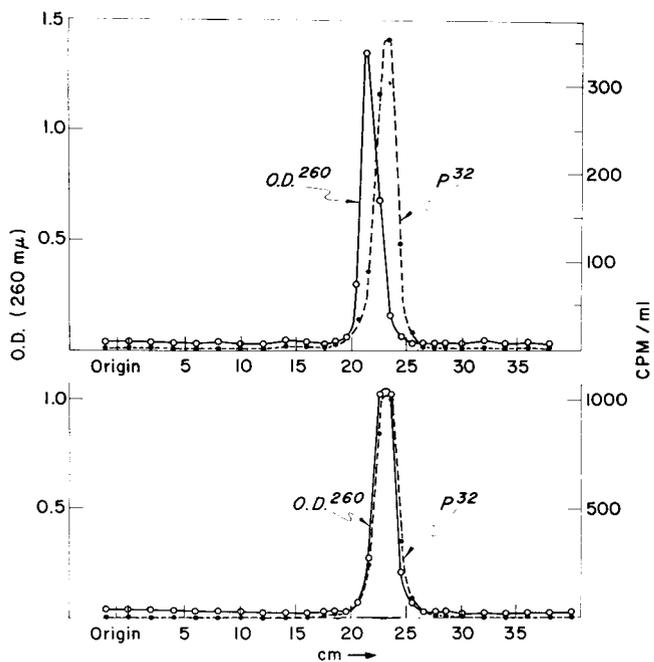


FIGURE 3. Starch column electrophoresis of P^{32} ribosome RNA. (a) RNA from T2-infected cells, given P^{32} between 5 and 7 min after infection. (b) RNA from control cells given a 2 min P^{32} pulse. In each case, electrophoresis was carried out for 10 hr at 8.0 v/cm, 6 ma in 0.03 M-tris buffer, pH 7.8.

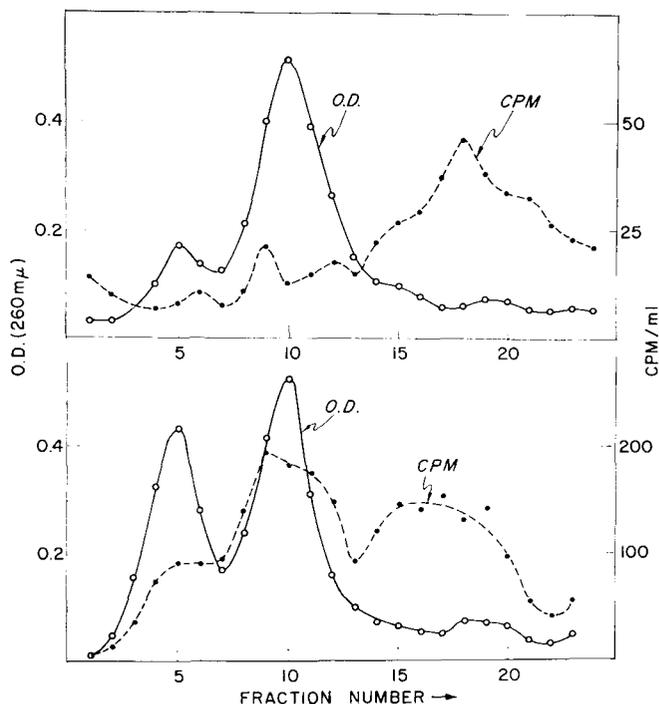


FIGURE 4. Sedimentation of P^{32} particle RNA. (a) RNA from T2-infected cells, exposed to P^{32} between 5 and 7 min after infection. (b) RNA from the control cells given a 2-minute P^{32} pulse. 0.25 mg RNA in 0.03 M-tris (pH 7.8) were layered on 4.4 ml of sucrose solution, with a concentration gradient from 3 to 20%. This was centrifuged for 8 hr at 37,000 rpm. Fractions were collected by piercing the lower end of the tube and collecting 10-drop fractions. To each fraction 1 ml 0.03 M-tris buffer was added; optical density at 260 $m\mu$ and P^{32} content were then measured.

cells emerged when ribosomal RNA synthesized during short (3 min) P^{32} pulses was examined by centrifugation in linear sucrose gradients. Figure 4 compares the profiles obtained in the two situations. The first peak in the optical density profile corresponds to the 23S component and the second to the 16S. The striking difference between the infected and control pulses is that little, if any, of the RNA synthesized in the T2-infected cells corresponds to the two main ribosomal components. There is very little concordance between the O.D. and radioactive profiles in the 23S and 16S regions. Most of the RNA formed subsequent to T2-infection appears to possess, by these methods of isolation, a sedimentation lying between 8 and 12S. In the non-infected control we note a considerable formation of RNA characteristic of the 23S and 16S varieties. It must, however, again be emphasized what is clearly evident from the two sets of curves of Fig. 4. The synthesis of the smaller size of RNA is *not unique* to the virus infected cell. The short P^{32} -pulse (Fig. 4b) readily exhibits its existence in the uninfected controls. These experiments reveal that the essential effect of infection with virus is a preferential suppression in the synthesis of the 23S and 16S RNA species.

In summary then, the experiments just described establish the existence of a T2-specific RNA as a

physically separable entity. The features which serve to distinguish it from normal ribosomal RNA may be listed as follows: a) a base ratio homologous to the viral DNA, b) metabolic instability, c) a higher electrophoretic mobility, d) a greater heterogeneity in size with an average sedimentation constant lower than 16S.

III. SEQUENCE COMPLEMENTARITY OF T2-DNA AND T2-SPECIFIC RNA

The procedures employed (zone electrophoresis and sedimentation) in the investigations just summarized led to the selective separation of T2-specific RNA. They, therefore, opened up the possibilities for further experiments relevant to an understanding of its nature. The fact that T2-RNA possesses a base ratio analogous to that of T2-DNA is of interest principally because it suggests that the similarity may go further and extend to a detailed correspondence of base sequence. The central issue of the significance and meaning of T2-RNA is whether or not this is, in fact, the case.

A direct attack on this question by complete sequence determinations was and is technically not feasible. However, the findings of Marmur (1960) and Doty *et al.* (1960) suggested the possibility for an illuminating experiment. These authors demonstrated

the specific reformation of double stranded DNA when heat denatured DNA is subjected to a slow cooling process. Such reconstitution of double stranded structures occurs only between DNA strands which originate from the same or closely related organisms. Presumably, the specificity requirement for a successful union of two strands reflects the need for a perfect or near perfect complementarity of their nucleotide sequences. We have here, then, a method for detecting the complementarity of nucleotide sequences in two strands of polynucleotide. The formation of a double stranded hybrid structure during the slow cooling of a mixture of two types of polynucleotide strands can be accepted as provisional evidence for complementarity of the bases of the input strands.

Hall and Spiegelman (1961) employed this procedure to examine for the complementarity of sequences between the T2-RNA and the T2-DNA. Purified T2-RNA was used in order to provide an optimal opportunity for the T2-RNA to combine with its DNA complement unhindered by non-specific interactions involving irrelevant RNA. Since the hybrid would have a lower density than uncombined RNA, a separation of the two should be attainable by equilibrium centrifugation in cesium chloride gradients (Meselson, Stahl, and Vinograd, 1957). To insure a sensitive and unambiguous detection of the hybrid, should it occur, double labeling was employed. The T2-RNA was marked with P^{32} , and the T2-DNA with tritium. Two isotopes emitting beta particles differing in their energies are conveniently assayed in each other's presence in a scintillation spectrometer. This device, coupled with the use of the swinging bucket rotor for equilibrium centrifugation, permits the actual isolation of the pertinent fractions along with a ready and certain identification of any hybrids formed.

Mixtures of P^{32} -labeled T2-RNA and H^3 -labeled single stranded T2-DNA were subjected to slow cooling. They were then put in CsCl and centrifuged to density equilibration. Figure 5 shows the optical density profiles and the distribution of tritium and P^{32} obtained from three preparations slow-cooled from different starting temperatures. Comparison of the profiles of tritium and P^{32} show that in all three cases slow cooling of the DNA and RNA produced a new peak of P^{32} approximately centered on the band of tritium (denatured DNA). This new P^{32} containing band must contain a DNA-RNA hybrid having approximately the same density as denatured T2-DNA. The amount of complex formed on cooling from the three temperatures was the same within experimental error. The three differ slightly, however, in the density of the complex relative to DNA.

Exposure to the slow cooling process is necessary since an uncooled mixture of T2-RNA and single stranded T2-DNA exhibited no P^{32} peak in the DNA

region when subjected to a centrifugal analysis. Furthermore, the presence of single stranded DNA is necessary during the cooling process. Double stranded DNA is unable to form hybrid with RNA under these conditions. Finally, the ability of T2-RNA to hybridize was found to be specific for T2-DNA. Thus, when the cooling process was carried with mixtures of P^{32} -labeled RNA of T2 and denatured DNA from heterologous sources (*Pseudomonas aeruginosa*, *E. coli*, and bacteriophage T5), no evidence of significant hybrid formation was observed. It is of interest to note that, although T5 has the same over-all base ratio as T2, no evidence of interaction of T2-RNA and T5-DNA was detected.

The data obtained in the course of this investigation showed that RNA molecules synthesized in bacteriophage infected cells have the ability to form a well-defined complex with denatured DNA of the virus. Further, this interaction is unique to the homologous pair as shown by the virtual absence of such complexes when T2-specific RNA is slowly cooled with heterologous DNA. The fact that T2-RNA and DNA do satisfy the specificity requirement must reflect a correspondence of structure between the two. Structural specificity of this order in single polynucleotide strands can only reside in specified sequences of nucleotides. It was concluded, therefore, that the most likely interrelation of the nucleotide sequences of T2-DNA and RNA is one which is complementary in terms of the scheme of hydrogen bonding proposed by Watson and Crick (1953).

IV. THE OCCURRENCE OF NATURAL DNA-RNA COMPLEXES IN THE *E. COLI* T2 COMPLEX

The fact that evidence could be provided for sequence complementarity between a specific RNA and its homologous DNA lends obvious support to the supposition that the normal process of transferring information from DNA to the protein synthesizing machine involves a mechanism whereby single stranded DNA serves as a template for the polymerization of a complementary ribopolynucleotide. 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.

Spiegelman, Hall, and Storek (1961) undertook to find such complexes, and the T2 *E. coli* system was selected as the most suitable in the initial search for native hybrid. The experimental devices employed were similar in essence to those used in the previous investigation on artificially formed complexes. These involved the use of double labeling and equilibrium centrifugation in cesium chloride gradients employing swinging bucket rotors. The T2-DNA was labeled with P^{32} by growth of the virus in a medium containing this isotope. T2-specific RNA was marked with tritium

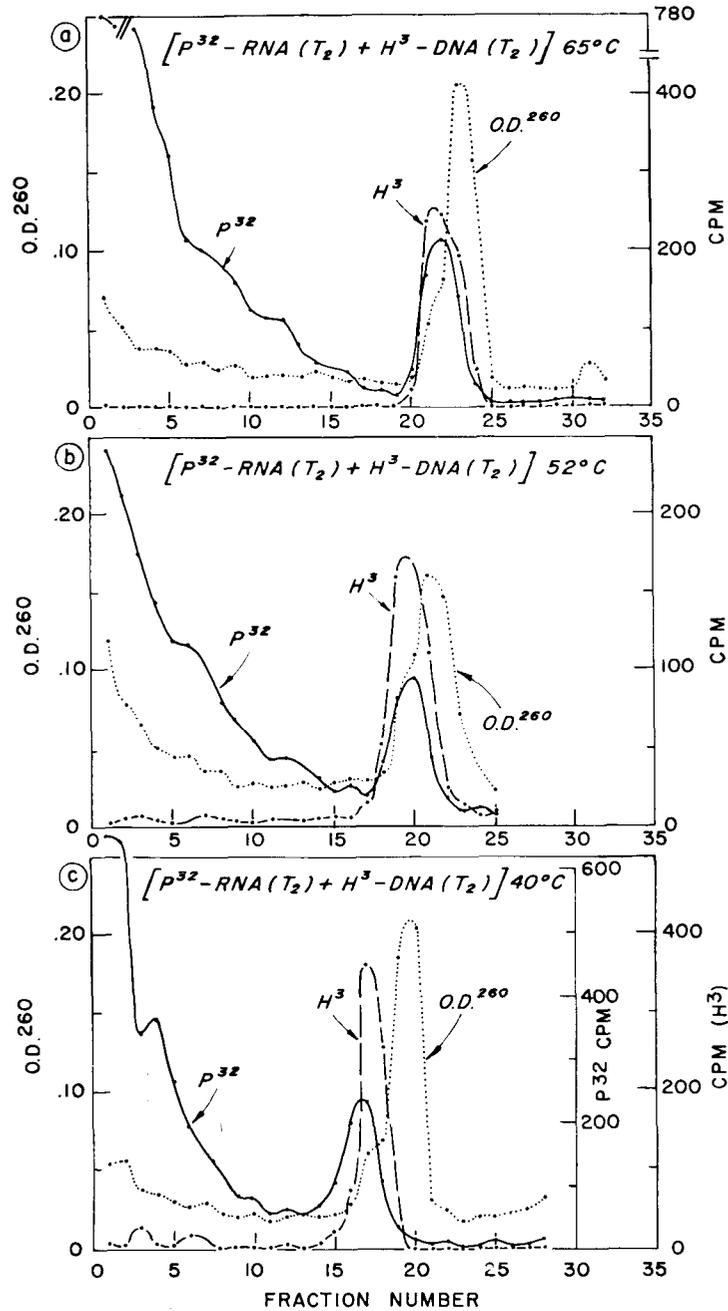


FIGURE 5. Formation of DNA-RNA hybrid at various temperatures. CsCl-gradient centrifugation analysis. P^{32} -RNA(T_2) ($14 \mu\text{g}$) and H^3 -DNA(T_2) ($6.5 \mu\text{g}$) were mixed in 0.6 ml 0.3 M NaCl and 0.03 M Na citrate, (pH 7.8); then the solution was immediately placed in the slow-cooling bath. Three identical solutions were made; (a) was placed in the bath at 65° , (b) at 52° , and (c) at $40^\circ C$. When the bath temperature reached 26° , CsCl and $25 \mu\text{g}$ T_2 -DNA were added to each solution; then they were centrifuged for five days at 33,000 rpm.

by introducing tritiated uridine. It is known that some uridine ends up as thymidine once DNA formation begins in the T_2 -*E. coli* complex. To avoid the complications this would introduce in identifying radioactive peaks, the initial search for hybrids was confined to the period (2-5 minutes) when no DNA synthesis can be detected. In a double label experi-

ment the presence of DNA-RNA hybrids would be signaled by the appearance of coincident peaks of the two isotopes. Further, these peaks should occupy a position in the cesium chloride gradient differing from those which characterize the densities of RNA and double stranded DNA.

A variety of procedures were surveyed for ob-

taining material suitable for the reliable detection of DNA-RNA hybrids by cesium chloride density equilibrium centrifugations. It was empirically established that removal of most of the protein was a necessary step prior to introducing the material into the cesium chloride. In the absence of this preliminary purification much of the nucleic acid, including hybridized material, was trapped in the protein layer found floating at the top of the gradient. The procedure ultimately adopted consisted essentially of the first few deproteinization stages normally (Marmur, 1961) employed for preparing DNA from bacterial cells. Two modifications were made. None of the steps designed to remove RNA is included. Further, at the alcohol precipitation stage centrifugation, rather than winding around a glass rod, is used to collect the nucleic acid. Such DNA preparations are, of course, heavily contaminated with RNA but serve well the intended purpose.

In experiments which employ P^{32} -labeled T2-DNA and tritiated uridine as the RNA marker, existence of hybrid would be detected as a tritium peak in the DNA region somewhat heavier than the P^{32} peak corresponding to the T2-DNA. The P^{32} peak should exhibit signs of bimodality as evidence of distribution of the T2-DNA between hybrid and unhybridized T2-DNA. Figure 6 describes the results of a typical

profile obtained by subjecting a nucleic acid preparation from a pulse labeled T2-infected cell to equilibrium gradient centrifugation. Three peaks are readily discernible. The lightest one corresponds to the input P^{32} -labeled DNA of T2. The optical density peak identifies the position of double stranded *E. coli* DNA put in as a marker. The tritium peak locates the hybrid. Furthermore, a shoulder makes its appearance on the heavy side of the P^{32} profile corresponding in position to the tritium peak region. The relative positions of the peaks and the bimodality of the P^{32} distribution are all consistent with what would be predicted from the existence of a DNA-RNA hybrid.

If it be assumed that a small amount of T2-DNA can be synthesized even in this early period of infection, another interpretation of the optical density and radioactive profiles can be entertained. This would presume the appearance of new H^3 -labeled T2-DNA, all in the form of single stranded material, along with the conversion of a portion of the input P^{32} -labeled DNA to single strands. To test for this possibility, the alkali lability of the tritium and P^{32} in relevant fractions of a number of experiments was examined. The same results were obtained in all cases and are exemplified by the data summarized in Table 1. Here, the two fractions indicated by arrows in Fig. 6

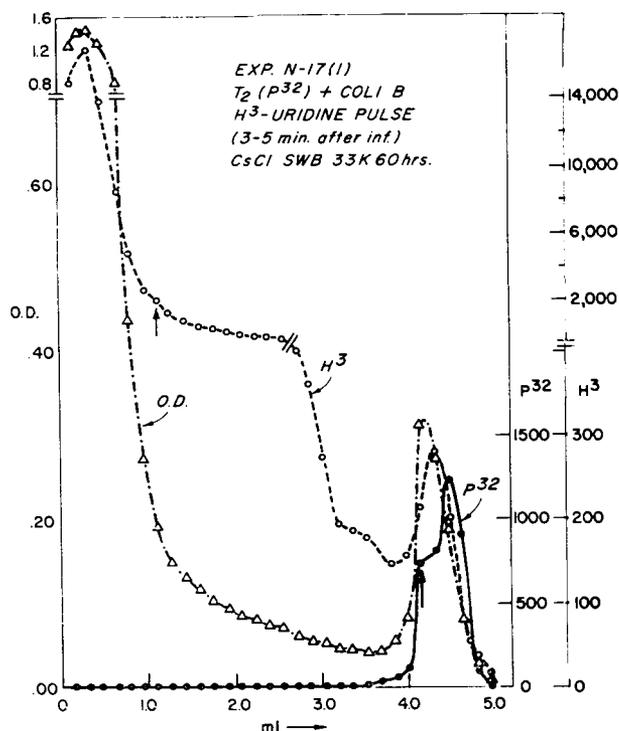


FIGURE 6. Equilibrium density centrifugation in CsCl of nucleic acid from T2-infected cells. Adsorption of phage was carried out at a multiplicity of 15 of undiluted P^{32} -labeled virus. Pulsing was done between 3-5 minutes after zero time with H^3 -uridine having a specific activity of $530 \mu\text{c}/\mu\text{M}$ at a level of $1 \mu\text{g}/\text{ml}$. Note that the radioactivity scale is expanded in the hybrid region.

TABLE 1. EFFECT OF ALKALI DIGESTION ON ACID PRECIPITABILITY

0.4 ml aliquots of Fractions 7 and 26, indicated by arrows in Fig. 6 were made 0.3 N with respect to NaOH and incubated for 24 hours at 30°C. Equivalent aliquots were held as controls under the same conditions. Following the incubation, the alkali was neutralized, carrier herring sperm DNA added and the contents precipitated with TCA. The precipitates were then washed and counted.

Fraction		Acid Precipitable Counts/ml.	
No.	Isotope	Control	After Alkali Treatment
26	H ³	230	5
	P ³²	735	720
7	H ³	1950	10
	P ³²	—	—

were subjected to alkali digestion, and the resultant effect on the acid precipitability of tritium and P³² was examined. Fraction 7 was included as a free RNA control. It is evident from the data in Table 1 that all of the tritium and none of the P³² counts are alkali labile. These data appear to eliminate the possibility that the displaced tritium peak can be ascribed to newly synthesized single stranded DNA. It is also evident that the displaced P³² cannot be ascribed to a conversion of the input viral DNA components to an RNA polynucleotide.

We may conclude, therefore, that a hybrid exists containing newly synthesized T2-specific RNA complex with some of the input P³² labeled DNA. It should be noted that hybrids involving the input DNA were also observed in pulses covering, in addition to the 2 to 5 minutes, 9 to 12 and 19 to 22 minutes after infection.

The existence of the natural hybrids constitutes another link in the chain of evidence supporting the simple complementary transcription mechanism mentioned earlier.

V. SELECTIVE SYNTHESIS OF INFORMATIONAL RNA IN NON-INFECTED CELLS

We now turn to the last question posed in the introductory paragraphs, namely the existence of complementary RNA in normal cells. The detection and study of the properties of complementary RNA formed in T2-infected cells was greatly facilitated by the fact that the larger ribosomal components are not synthesized. This advantage is not present in normal uninfected cells, which consequently complicates the search for normal informational RNA. That it is, nevertheless, feasible is suggested by the experiments

of Yčas and Vincent (1960) with yeast. These authors used P³² in a manner comparable to the procedures of Volkin and Astrachan and, despite surprisingly long pulses, were able to detect formation of a fraction with a high metabolic turnover and possessing a base composition analogous to yeast DNA. In a preliminary report, Astrachan and Fischer (1961) suggest that very short P³² pulses lead to a distribution of labile in the RNA synthesized, which indicates the synthesis of RNA mimicking the base composition of DNA.

It would clearly be of great advantage if a situation 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 formation of the informational variety. The possibility that a situation of this sort might, in fact, be realizable was suggested by studies on RNA and protein synthesis during passage from fast to slow growth. Many of these investigations have been extensively reviewed in the present symposium by Neidhardt, Maaløe, and Schaechter and we need not, therefore, go into any details.

Several features emerged which encouraged us to look more carefully into such transitions. It has been known for some time that the RNA content per cell is positively correlated with its growth rate. Since the bulk of the RNA is ribosomal, it would mean that cells with higher growth rates possess more ribosomes. Consider, then, the situation when one subjects a culture to a "step-down" transition by transferring cells from a rich (e.g., Penassay) to a synthetic medium. The growth rate is decreased by a factor of two. But more important, the cells have more ribosomes than they can use. From the point of view of selective advantages it is not surprising, therefore, to find that such step-down transitions cause a rather dramatic cessation of net RNA synthesis. Nevertheless, protein synthesis proceeds for a while at near normal rates. From the viewpoint of relative rates of net protein and RNA synthesis, such cultures are analogous to T2-infected cells. It seemed not too unlikely that whatever RNA synthesis was occurring was restricted to the variety immediately necessary for the fabrication of new protein molecules. It was possible that this variety was the normal informational RNA for which we were searching.

Hayashi and Spiegelman (1961) instituted an investigation designed to see whether the above expectations were realizable. Base compositions of RNA synthesized in "step-down" cultures were examined. In all cases, such cultures were attained by transfer from a complete (Penassay) to a synthetic (Med. C. of Roberts *et al.*, 1957) medium. Base ratios were determined by procedures similar to those used in the study of the T2-*coli* system. P³² pulses were made and the ribonucleic acid

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

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 P³². 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, counted and O.D. at 260 m μ determined. The numbers given are derived from the distribution of the counts and isotope dilution. 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.

Organism	Minutes after Transfer	Moles Per cent					
		C	A	U(T)	G	% GC	Pu/Pyr
<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

isolated and purified, subjected to alkaline digestion, and the distribution of counts in the resultant 2'-3' nucleotides determined. Table 2 summarizes experiments with three different organisms in which the P³² pulse was carried out at various periods subsequent to the transitional transfer. It will be noted that in each case the RNA synthesized during the transition period mimics the homologous DNA in its per cent GC and purine to pyrimidine ratio. What is even more remarkable is the length of time during which this selective synthesis of complementary RNA continues. Thus, in the case of *Ps. aeruginosa*, even 60 minutes after the transfer a major fraction of the RNA formed is homologous to its DNA.

VI. THE SIZE DISTRIBUTION OF INFORMATIONAL RNA FORMED IN NON-INFECTED CELLS

The data summarized in Table 2 appeared to confirm our expectation that informational RNA is preferentially synthesized in step-down cultures. It was of obvious interest to continue this investigation and see whether the other properties of informational RNA revealed by the study of the T2-*E. coli* complex obtained here as well.

Size distributions of the RNA synthesized were examined by the usual procedure of labeling and subjecting the purified RNA to a swinging bucket analysis on a sucrose gradient. Figure 7 gives the results obtained with *E. coli* exposed to a 30 min "pulse" of H³-uridine during a step-down transition. The optical density profile identifies readily the 23, 16, and 4S RNA components pre-existing in the cell. A similar period of labeling in a normal culture

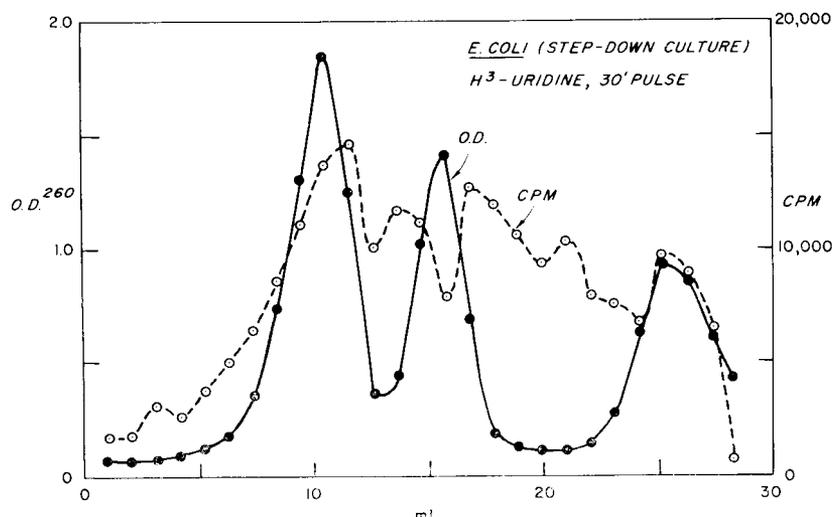


FIGURE 7. Swinging bucket analysis in 3-20% sucrose gradient of phenol purified RNA. Cells were exposed to H³-uridine for 30 minutes during transition from complete to synthetic medium. Closed circles identify pre-existent and open circles newly synthesized RNA.

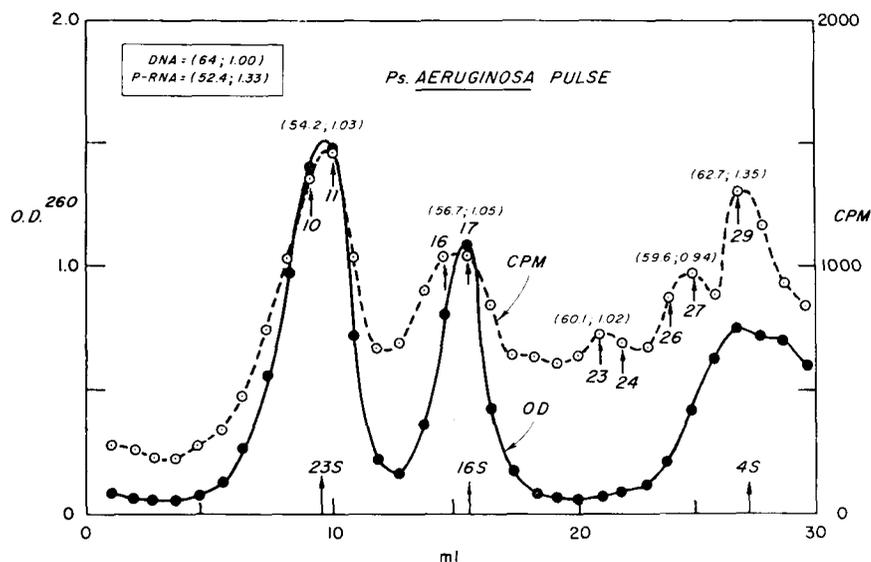


FIGURE 8. Swinging bucket analysis in 3-20% sucrose gradient of phenol purified RNA. Cells were exposed to a 3 minute P^{32} -pulse 5 minutes after they were transferred from complete to synthetic medium. Closed circles identify pre-existent and open circles newly synthesized RNA. The first number in parentheses represents per cent GC and the second the ratio of purines to pyrimidines.

Arrows indicate the fractions taken for base composition analysis.

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

Conditions of experiment and analyses similar to those described in Table 2. The fractions taken are those indicated by arrows in Fig. 8. P-RNA means purified ribosomal RNA, and the base composition was obtained from UV absorption data of the nucleotides.

Fraction # (Fig. 8)	Region	Moles Per cent					
		C	A	U(T)	G	% GC	Pu/Pyr
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

transferred from synthetic to synthetic medium would have resulted in virtually complete coincidence of the radioactivity and optical density profiles. Here we see little, if any, agreement between the two.

It was clearly desirable to extend our information on the RNA being synthesized, with particular reference to the base compositions of the various size ranges of RNA observed. For obvious numerical reasons and other technical considerations, *Ps. aeruginosa* was chosen for this more detailed analysis. A step-down culture was subjected to a P^{32} pulse, and

the ribonucleic acid examined centrifugally in a sucrose gradient. The optical density and radioactivity profiles are given in Fig. 8. The fractions indicated by arrows were analyzed for base compositions. For purposes of ready comparison, numbers corresponding to the per cent GC and purine to pyrimidine ratio which characterize each region are recorded in parentheses. Further details on the base ratio analyses are given in Table 3. Comparison of the parameters reveals that DNA-like RNA of all size classes have been synthesized, confirming the findings with *E. coli* (Fig. 7). As one proceeds to the smaller size ranges (16S-6S) the homology between the RNA and DNA becomes excellent.

Metabolic instability is a diagnostic characteristic of the T2-RNA, and it was of obvious interest to see whether this feature was possessed by the complementary RNA synthesized in the transition period. Accordingly, a culture of *Ps. aeruginosa* was subjected to a P^{32} pulse in exactly the same manner as detailed in the experiment of Fig. 8. However, twice as much radioactivity was used. Following the 3 minutes of labeling, the culture was centrifuged, washed, and re-introduced into the same medium containing P^{32} . It was then allowed to chase for 0.7 generations. Figure 12 describes the optical density and radioactivity profiles obtained when purified RNA from this preparation was centrifuged in the usual way. Here again individual fractions were taken for base ratio determinations. The numbers in parentheses give the results in terms of per cent GC and purine to pyrimidine ratios.

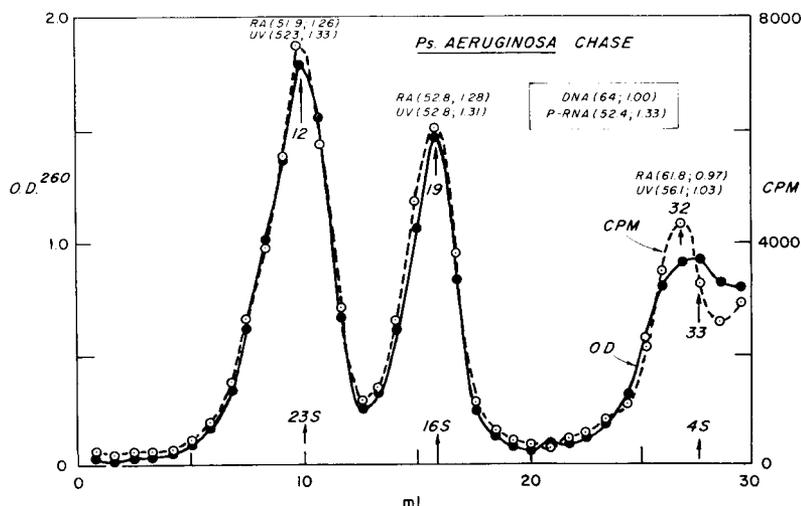


FIGURE 9. Swinging bucket analysis in a 3–20% sucrose gradient of phenol purified RNA. Cells represent an aliquot taken from the experiment of Fig. 8 and exposed to a “chase” for 0.7 generations in non-radioactive synthetic medium. The first number in parentheses represents per cent GC and the second, ratio of purines to pyrimidines. The values found by both UV absorption and radioactivity are given. Arrows indicate fractions subjected to base composition analysis.

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

A culture treated like that used in the experiment of Table 3 and Fig. 8 was taken after the 3 min P^{32} -pulse, washed, and allowed to grow for 0.7 generations in 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 Fig. 9. 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 hours.

Fr #	Region	<i>Ps. aeruginosa</i> Moles per cent											
		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

Table 4 provides further details on the base ratios determined by both the ultraviolet absorption derived from the added carrier and the distribution of radioactive counts among the 2'-3' nucleotides as eluted from a Dowex column. Comparison of Figs. 8 and 9 provides clear evidence of the metabolic instability of the heterogeneous RNA synthesized during the transition period. The interval of chasing eliminated almost completely the discordancies between the optical density and radioactivity profiles seen in the initial pulse (Fig. 8). Furthermore, as is evident from Table 4, the base ratios of the labeled RNA in the 23S

and 16S region are now typically ribosomal. There is excellent agreement between the base ratios determined by the distribution of radioactive counts and ultraviolet absorption.

It is of interest to note that despite the fact that the chase extended for a period of 0.7 of a generation, there is still some discrepancy in both the profiles of Fig. 5 and the base compositions in the 4S regions (Table 4). This may be a reflection of the difficulty of completely removing informational RNA. It would be consistent with a mechanism which involves a comparatively rapid breakdown of the larger informa-

tional RNA pieces to 4S size and a slower conversion of these to the level of nucleotide derivatives.

VII. HYBRIDIZABILITY OF INFORMATIONAL RNA FROM NORMAL CELLS WITH HOMOLOGOUS DNA

The experiments described thus far with non-infected cells established that "step-down" cultures preferentially synthesized a type of RNA which was heterogeneous in size, was metabolically unstable, and possessed an over-all base ratio which was analogous to its homologous DNA. These are features expected of informational RNA. To complete the identification, it was necessary to test for sequence complementarity by the hybridization procedure. An examination of the hybridizability of complementary RNA to homologous and heterologous DNA was undertaken by Spiegelman, Doi, and Yankofsky (1961). We cite here only 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 H^3 -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 $55^\circ C$.
- 6) The resulting mixtures were then subjected to equilibrium centrifugation in CsCl gradients according to the methods described by Hall and Spiegelman (1961).

Figure 10 shows the outcome of a hybridization

carried out between single stranded *E. coli* DNA and 8-12S H^3 -RNA labeled 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 *coli* DNA. That the interaction is specific is shown in Fig. 11 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 was labeled with H^3 -uridine during a step-down transition. Figure 12 shows the outcome of a hybridization carried out with homologous single stranded DNA and H^3 -RNA removed 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 10 gamma of RNAase for 15 min at room temperature; and then carrier DNA was added and the material reprecipitated, washed, and re-counted. It will be noted that most of the counts corresponding to free RNA are almost completely removed by the RNAase treatment. However, the counts in the region of the hybrid are obviously much more resistant to RNAase.

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 established that RNA molecules preferentially synthesized in "step-down" cultures possess base sequences complementary to their homologous DNA.

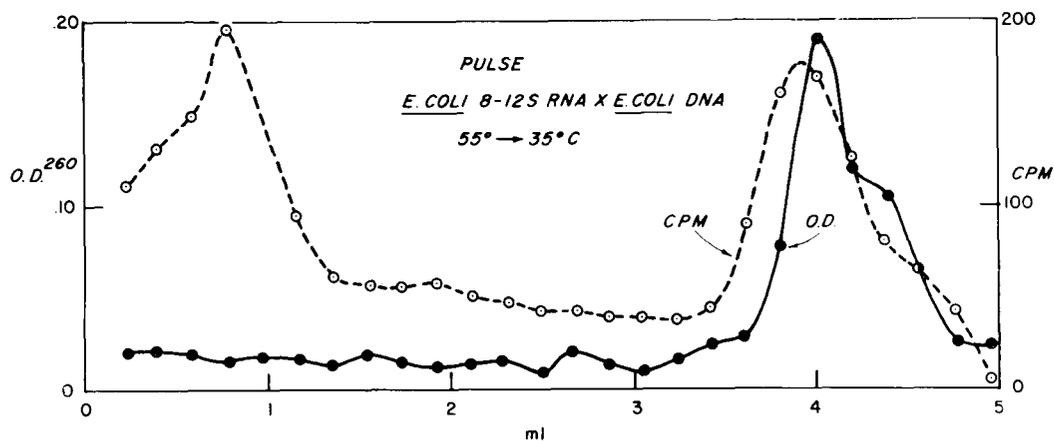


FIGURE 10. Equilibrium density centrifugation (33K 60 hours) in CsCl. A mixture of H^3 -RNA (8-12S) from an *E. coli* "step-down" culture slow cooled with single stranded *E. coli* DNA. Double stranded *E. coli* DNA was added as a marker and is represented by the shoulder on the light (right) side of the main O.D. peak.

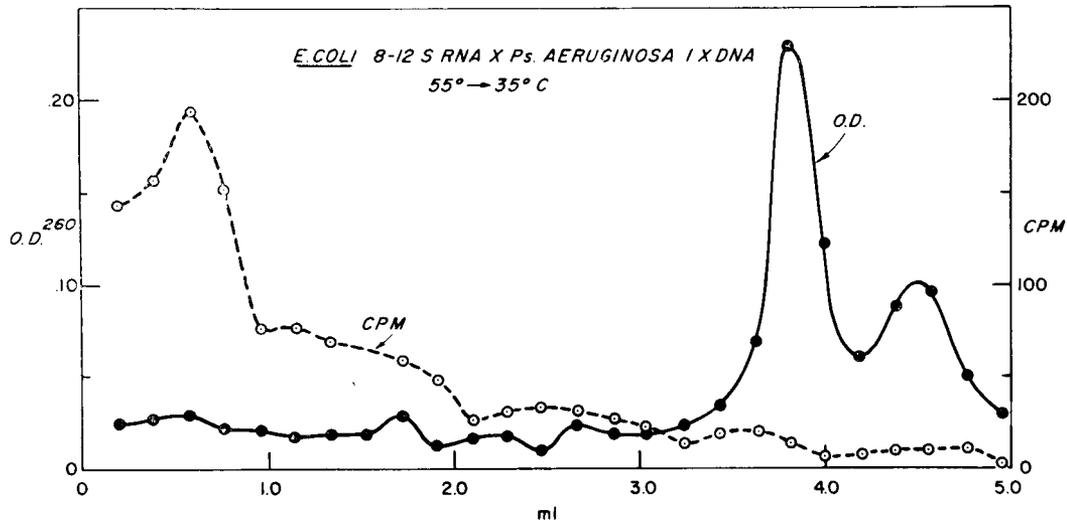


FIGURE 11. Equilibrium density centrifugation (33K 60 hours) in CsCl. A mixture of H^3 -RNA (8-12S) from an *E. coli* "step-down" culture slow cooled with single stranded DNA from *Ps. aeruginosa*. Double stranded *E. coli* DNA was added as a marker and is represented by the second peak to the right.

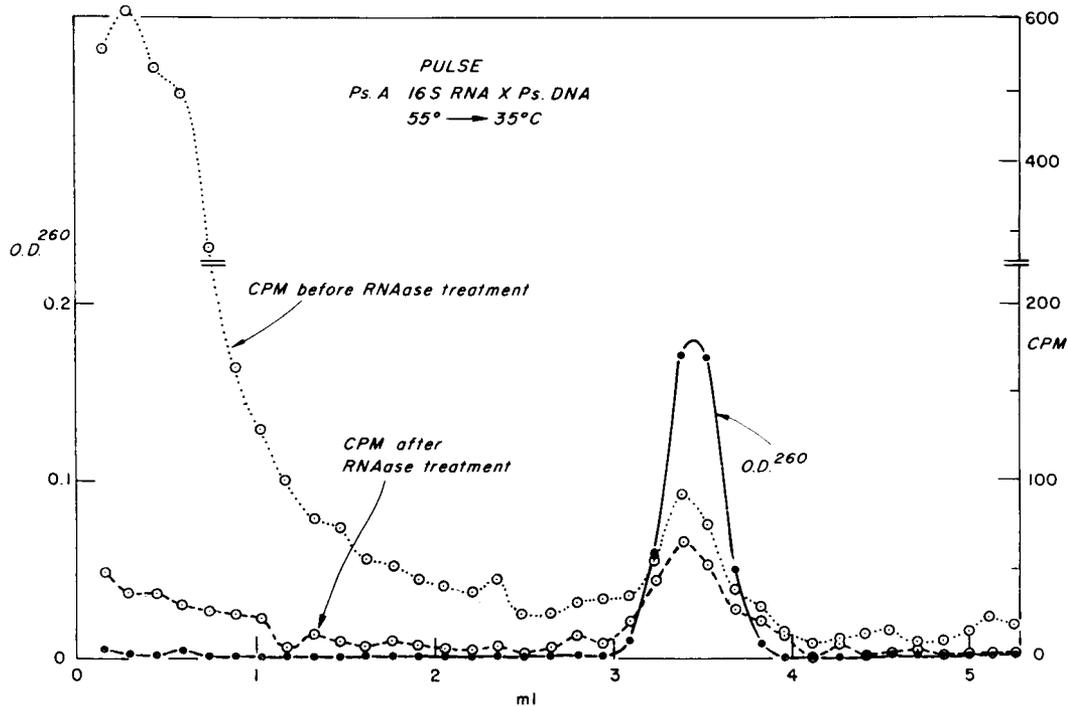


FIGURE 12. Equilibrium density centrifugation (33K 60 hours) in CsCl. A mixture of H^3 -RNA (16S) from a *Ps. aeruginosa* "step-down" culture slow cooled with single stranded DNA from *Ps. aeruginosa*. No marker was added. Open circles-dashed line gives the effects on the cpm of treatment of the indicated fractions with RNAase prior to precipitation and counting.

VIII. DISCUSSION

We may perhaps begin with a few words on terminology. The terms "complementary" and "informational" have been used to describe the RNA molecules with which we are concerned. It is evident from the

experiments discussed that these terms have well-defined operational definitions. A given RNA molecule is defined as falling within the informational class if its base ratio is homologous and its sequence is complementary to a specific DNA molecule. At present

the most sensitive test for complementarity of sequence is the hybridization experiment of Hall and Spiegelman (1961). Every "complementary" RNA is "informational" in at least one sense. Even if it is a complementary copy of a nonsense DNA sequence, it still contains the information necessary to specify the order of the bases.

It is important to emphasize that the word "informational" is not proposed as a substitute for the term "messenger" introduced in the elegant experimentations and theorizations of Jacob and Monod (1961). 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 serve a messenger function. It is conceivable, as is indeed implicit in the operon theory of Jacob and Monod (1961), that informational RNA molecules will be found which serve regulatory rather than programming functions.

Before concluding it may be useful to discuss some problems and implications which arose during the investigations described and which have not received explicit mention.

A. SIZE AND STABILITY OF INFORMATIONAL RNA

As our experience with informational RNA accumulated, it became more and more evident that this type of RNA is uniquely susceptible to degradation in extracts. Even highly purified preparations, obtained from either T2-infected cells or "stepped-down" cultures, were much more fragile than ribosomal RNA carried through the same procedures. In a sense, the behavior of informational RNA reminds one of the apparent inherent instability of the synthetic polypyrimidines (see discussion of Rich, 1958). The underlying reason may well be the same, and there is a pressing need for an understanding of its chemical basis.

In any event, we became increasingly careful in the methods used to prepare this type of RNA. By the time the experiments involving informational RNA from normal cells (section VI) were performed, procedures had been devised which minimized contact of the newly synthesized RNA with enzymatically active extract. It will be noted from the results described in this section that there is clear evidence in these cases of informational RNA, 16S and larger, possessing hybridizing ability.

These observations led us to consider the possibility that the smaller, and more homogeneous size, deduced from the earlier experiments with the T2-infected cells (section II), might have been a consequence of the fact that we had not at that time understood fully the instability problem. Experiments were therefore undertaken by Sagik, Green, and Spiegel-

man (1961) to re-examine the size distribution of T2-specific RNA using the newer procedures. It was indeed found that T2-complementary RNA showed a much more heterogeneous distribution of sedimentation constants than that which characterized the earlier experiments. RNA hybridizable with T2-DNA was found in sizes ranging from 23S-8S.

Our experience to date leads to the conclusion that informational RNA is extremely heterogeneous in size. Left unresolved is the question of whether the small size ranges observed are real or artifacts of unavoidable breakdown.

These considerations raise another issue of practical significance. It is evident that "soluble" RNA is very likely to contain small informational components as contaminants of the presumed transfer RNA molecules. In this same connection it should be noted that the chasing experiment of Fig. 9 did not suffice to completely remove informational RNA from the 4S region even after 0.7 generations. Stimulatory effects on amino acid incorporation with so-called "soluble" RNA are obviously open to more than one interpretation with respect to the responsible agent.

B. GENESIS OF THE MAJOR RNA COMPONENTS

The question naturally arises of the nature of the major RNA constituents, the base ratios of which bear no obvious relation to homologous DNA's. Two hypotheses can be entertained as to their origin. One is they are complementary in the sense of being synthesized by a DNA mediated mechanism. The second assumes that DNA is not involved. In this case, the mechanism of their formation might involve an RNA template as a guide or no template, the polymer being put together much as polysaccharides are formed.

It might perhaps seem a simple matter to settle this by examining for hybridizability of the relevant RNA to DNA. In the course of the present studies, experiments have been performed in an attempt to detect hybrid formation with 23 and 16S ribosomal RNA. None has been found. On the basis of the data available we can state that on a per μg basis, the hybridizing ability of ribosomal RNA is 10-100 times poorer than information RNA obtained by the methods described. However, this does not serve to eliminate the possibility of hybrid formation with ribosomal RNA. The numerology of the situation makes it technically difficult to obtain a definitively negative answer. Consider the 23S molecule, which is the most favorable. Its molecular weight of 1.6×10^6 represents approximately .01% of the bacterial genome. If there is only one section of the DNA concerned with its synthesis, then this is the only portion which will hybridize. To settle questions of this nature one will have to push the sensitivity of hybrid detection two orders of magnitude beyond

that used to date. This is technically difficult but by no means impossible.

C. ENZYMOLOGICAL IMPLICATIONS

The transcription mechanism supported by the data described in the preceding sections would require a DNA dependent enzymatic mechanism for polyribonucleotide synthesis. Early evidence suggesting the existence of such a pathway emerged from a study (Spiegelman, 1958) of a cell-free system derived from *E. coli*. These preparations possessed considerable capacity to synthesize polyribonucleotide. It was routinely possible to obtain between 10- and 20-fold increases of polyribonucleotide. The observed synthetic activity exhibited a requirement for riboside-triphosphates, and Mn, and was severely inhibited by treatment with DNAase. The last year has witnessed a notable advance in our understanding of the details of this reaction. Recently a number of laboratories (Weiss, 1960; Hurwitz, Bresler, and Diring, 1960; Stevens, 1960; and Ochoa, Burma, Kroger, and Weill, 1961) have independently achieved a considerable purification of an enzyme which synthesizes polyribonucleotide and requires riboside triphosphates and DNA. Furthermore, the base ratio of the polyribonucleotide synthesized bears a striking homology to that of the DNA used as a primer (Furth, Hurwitz, Goldmann, 1961; Weiss and Nakamoto, 1961). The details of these investigations will undoubtedly be thoroughly discussed by Hurwitz who has contributed so much to this area.

IX. SUMMARY AND CONCLUSION

The present paper records our attempts over the past three years to gain an understanding of the nature of the agent which allows the genome to exert its control over macromolecular synthesis. The direction taken by the series of investigations described was greatly influenced by two factors. One was the assumption that the intermediary between the DNA and the protein synthesizing mechanism must be a polyribonucleotide. The second was the observation of Volkin and Astrachan which suggested that an RNA homologous to the viral DNA was preferentially synthesized in an infected cell.

Before the Volkin-Astrachan deduction could be accepted as a departure point for a more extensive search for this type of RNA, its existence had to be proved. This was accomplished by selectively separating the newly synthesized RNA from the bulk of the RNA of the cell. Separation was achieved by both zone electrophoresis in starch columns and centrifugations in sucrose gradients. In addition to possessing a base composition homologous to T2-DNA, T2-RNA was found to have a lower average size and higher electrophoretic mobility. It was further shown that T2-specific RNA was ribosome-bound but with a linkage

much more sensitive to disruption than normal ribosomal-RNA.

Having established T2-specific RNA as a physical entity and provided methods for its selective enrichment, it was possible to go to the next stage of the investigation and inquire into the meaning and significance of the similarity of base ratios between it and T2-DNA. This was done in terms of attempts at hybrid formation. It was possible to show that RNA-DNA complexes were indeed formed when mixtures of single stranded T2-DNA and purified T2-specific RNA were subjected to slow cooling. The success of the hybridizing experiments suggested immediately that the original observation 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.

The next obvious step was to look for the presence of native complexes in systems actively synthesizing enzyme. Using detection procedures very similar to the ones employed in artificially producing hybrids it was possible to exhibit evidence for the existence of natural DNA-RNA hybrids in the *E. coli* T2 system.

Finally came the question of universality. The principle of biological unity would suggest that the mechanism of information transfer in normal uninfected cells should also involve a complementary RNA. In an attempt to provide evidence for this belief attention was turned to cultures subjected to a transition from a rich medium, which supported fast growth, to a synthetic one in which the growth rate was lower. Evidence had accumulated which led us to believe that cells undergoing such a shift were analogous in their relative RNA and protein synthesizing capacities to a T2-infected cell.

The expectation that such cells were preferentially synthesizing complementary RNA was fully realized. It was possible to establish with three different organisms the existence of an RNA in normal cells having all of the properties which had been established for the T2-specific RNA. This normal informational RNA exhibited a base ratio analogous to its homologous DNA, was metabolically unstable, very heterogeneous in size, and possessed the ability to hybridize specifically with its homologous DNA.

It would appear from the results summarized here and those reported from other laboratories at this Symposium that the search for the informational intermediary has been successful. All the data are happily consistent with the simplest transcription-mechanism mentioned in the introductory paragraphs, i.e., the informed intermediary is a complementary polyribonucleotide of its "parental" DNA.

ACKNOWLEDGMENTS

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DISCUSSION

HOTCHKISS: In this interesting work of Dr. Spiegelman's laboratory, and other recent work elsewhere, what is often spoken of as single stranded DNA plays an important part. I feel that it would be more appropriate to refer to this material as "heat-denatured DNA" when this is the way it has been made. Marmur, Doty, and Lane have made a most significant contribution in showing that certain types of heating will reduce the ordered double-strandedness of DNA, and also that some degree of double-strandedness can be restored by heat treatment. But with reactive molecules of such size and complexity it is unrealistic to picture or describe them as idealized single strands capable of expressing their specific affinities only toward *other* strands of DNA or RNA. With Dr. Muriel Roger of our laboratory we have reported distinct indications that heat-denatured pneumococcal transforming DNA collapses without loss of molecular weight, as though small portions of the molecule remain doubly-stranded. Size, weight, viscosity, and biological affinity and activity are in keeping with this picture for denaturation of material that does not contain chainbreaks, and it would fit that portion of Marmur's material which is capable of "renaturation." If our picture is appropriate, then denatured DNA will contain in different particles and different preparations variable-length regions of single stranded chains. This possibility would have considerable bearing on the stoichiometry of its ability to bind RNA or polynucleotides, and further, on its capacity to bind large or small RNA components. Such possibilities might be systematically exploited by making different kinds of heat-denatured DNA for such studies.