acid; CTP^{32}, ATP^{32}, UTP^{32}, and GTP^{32} for the triphosphates of cytidine, adenosine, uridine, and guanosine labeled with P^{32} in the ester phosphate; Cp, Ap, Up, and Gp for the 2′,3′-nucleoside monophosphates of the bases listed above; PP for inorganic pyrophosphate; Tris, tris-(hydroxymethyl)-aminomethane; TCA, trichloroacetic acid; cpm, counts per minute.

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*THE ENZYMATIC SYNTHESIS OF RNA: COMPLEMENTARY INTERACTION WITH DNA**

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The chemical properties of the polynucleotides synthesized by the DNA-primed RNA polymerase have been described in two previous communications.[1, 2] It has been shown that the average composition and the average nearest-neighbor frequencies of enzymatically synthesized RNA and DNA primer are identical. In view of this information and of the results communicated here, we have named the product of this reaction "Complementary RNA" (C-RNA).[3] It is our working hypothesis that C-RNA is structurally and functionally similar to "messenger" RNA,[9, 10] and that the biosynthesis of C-RNA is an initial step in that sequence of reactions in which DNA asserts the organism's potentialities for protein synthesis. Consequently, it is of considerable interest to discover whether higher-order DNA nucleotide *sequence* is translated with any degree of fidelity in RNA and to describe

in greater detail the manner in which the DNA template is utilized during RNA synthesis. In this communication, we describe the results of experiments along these lines.

Materials and Methods.—DNA: T2 DNA was isolated from bacteriophage by phenol extraction.[12] *Pseudomonas fluorescens* DNA, used as a density reference marker in analytical CsCl gradient centrifugation, and *Escherichia coli* DNA were prepared by a method described elsewhere.[13] DNA from testes of the sea urchin *Strongylocentrotus drobachiensis* was isolated according to the method of Simmons.[14] Bacteriophage T2 DNA, labeled with tritium, was prepared as follows: *E. coli* were grown as described by Kozloff.[15] At late log phase, T2 phage and H^3-labeled adenine were introduced into the medium, and aeration was continued for 4 hr. $MgSO_4$ to 0.005 *M* and 1 μg/ml crystalline pancreatic DNAase I were added. After incubation at 37°C for 1 hr, the entire culture was filtered through celite, and phage was purified from the filtrate.[16] DNA was prepared from the phage by phenol extraction.[12]

The synthesis of C-RNA on a bacteriophage T2 DNA template was carried out as described in the preceding communication.[2] The total volume of the reaction mixture was adjusted to allow incorporation of 50 to 100 mμmoles of labeled substrate into RNA in the presence of 150 to 300 mμmoles of primer DNA-P. The specific activity of labeled nucleotides was reduced to conform with the requirements of the centrifugation experiments.

Isolation of DNA and C-RNA for CsCl gradient centrifugation: Procedure A. The enzymatic synthesis of RNA was terminated by adding an equal volume of 90% redistilled phenol, and shaking 30 min. The phenol extraction was repeated. The aqueous layer containing RNA and DNA was then extracted two to three times with ether to remove dissolved phenol, and residual ether was removed with a stream of N_2. NaCl was added to 0.2 *M*, and CTAB[17, 18] to a final concentration of 1 mg/ml. A precipitate containing DNA and RNA formed immediately. The mixture was allowed to stand for 15–20 min and centrifuged. The precipitate was then washed twice with 0.2 *M* NaCl, and finally dissolved in a small volume of 2 *M* CsCl. All the above steps were carried out at room temperature. Under these conditions, we found that 90% or more of the enzymatically prepared RNA-P^{32} was recovered and that it contained 10 to 20% TCA-soluble nucleotides. The recovery of DNA was less complete, some loss from the aqueous phase occurring in the phenol extraction. However, we did not find it necessary to recover any of that material for the experiments reported here.

Procedure B. Phenol extraction was performed as above. The entire aqueous phase was then reduced under vacuum, adjusted to 7.9 molal CsCl, 0.01 *M* Versene, pH 6.5 in a final volume of 3 ml, and centrifuged at 16°C, 35,000 rpm, for 48 hr. The material which accumulates at the bottom of the tube in the CsCl gradient contains the bulk of the RNA, well separated from DNA. From this fraction, RNA was precipitated as follows: to 0.7 ml CsCl solution, 0.4 ml 0.01 *M* Tris, pH 7 was added, followed by 2.2 ml ice-cold ethanol. The turbid suspension was left standing at room temperature for 20 min, centrifuged, and washed twice with 70% ethanol (1 molal in CsCl). The RNA was then dissolved in the solvent appropriate to the experiment in question.

CsCl density gradient centrifugation in a bucket rotor: Solutions of DNA and RNA in 7.9–8.4 molal CsCl were centrifuged in SW 39 rotors at 35,000 rpm. Three ml of CsCl solution were placed in 5-ml lusteroid centrifuge tubes and overlayered with 2 ml of light liquid paraffin. For the experiments shown in Figures 4 and 5, temperature was controlled (in a Spinco model E analytical ultracentrifuge) within ±0.1°C. For the experiments shown in Figure 2 and 3, the temperature was near 0°C. but was not measured. Centrifuge runs were terminated after 65 to 90 hr, and fractions were collected in a manner similar to that described elsewhere.[19] Each fraction was assayed for acid-precipitable counts and absorbance at 260 mμ.

Analytical methods: Acid-precipitable RNA-P^{32} was determined as described previously.[2] Acid-precipitable DNA-H^3 was determined as follows: nucleic acids, together with exactly 2 mg carrier yeast RNA, were precipitated in 5% TCA and centrifuged, and the supernatant fluid was removed. The residue was dissolved in one drop of concentrated NH_4OH, quantitatively transferred to a planchet, dried, and measured in a windowless gas flow counter.

Properties of RNA synthesized on a phage T2 DNA template: The ultraviolet absorption spectrum of one preparation of T2-C-RNA prepared as above is shown in Figure 1. It has λ_{max} at 258 mμ and λ_{min} at 232 mμ. This material is macromolecular and very heterogeneous, having an

average sedimentation constant $s_{20,w}$ of 5.5*s*, measured at $c = 0.0017\%$ in 0.1 *M* NaCl, 0.01*M* Tris, pH 7. (By comparison, the "messenger" RNA of *E. coli* has a sedimentation constant of $8s^{20}$.) Accordingly, whatever the molecular configuration of C-RNA, this sample must have a much lower average molecular weight than the T2 phage DNA used as a template. We do not know whether this reflects an intrinsic property of RNA polymerase acting *in vitro* or whether it is merely due to degradation by enzymes contaminating the partially purified RNA polymerase.

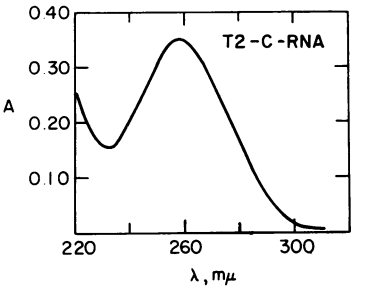

FIG. 1.—Ultraviolet absorption spectrum of T2-C-RNA in 0.01 *M* Tris, pH 7. Absorbances measured in 0.7-ml capacity cuvettes on Beckman DU spectrophotometer.

Results and Discussion.—(1) *Does C-RNA remain attached to the DNA template?* The answer to this question is given by two experiments which show that labeled RNA and DNA are almost completely separated from each other in a CsCl density gradient. In Figure 2, the CsCl gradient centrifugation of the nucleic acids extracted from a T2-C-RNA-P^{32} synthesis is analyzed. It is evident that RNA-P^{32} has accumulated at the bottom of the centrifuge tube and that only a small peak of RNA-P^{32} (less than 3% of the total) can be detected near that portion of the solution which, judged by absorbance measurements, contains the DNA. Figure 3 shows the corresponding experiment in which unlabeled C-RNA has been synthesized on a template of phage T2 DNA-H^3. As in Figure 2, the peak of optical density at the bottom of the tube is due to C-RNA, but there is no sign of a complex with tritiated DNA. The two experiments show that the two nucleic acids are almost completely separated from each other. At present, it is not possible to decide whether the small fraction of C-RNA associated with the DNA band represents C-RNA trapped on the DNA template or whether it is simply due to incomplete disaggregation at some stage of the preparation.

Only two steps have intervened between the RNA synthesis and the gradient centrifugation: phenol extraction at 25°C and precipitation with ethanol or CTAB. As used here, neither step involves even slightly denaturing conditions. Thus, if a complex between the bulk of the C-RNA and its DNA template has been disrupted, we can say with certainty *either* that it is very much more labile than the DNA double helix *or* that it involves a third component (e.g., protein) which is removed during the phenol extraction and nucleic acid precipitation.

(2) *Can DNA-C-RNA complexes be formed?* We have closely followed the elegant method of Hall and Spiegelman[11] in answering this question. Figure 4*a* shows that when T2-DNA and T2-C-RNA are heat-denatured and then "annealed" at 40°C, DNA-C-RNA complexes do form. On the other hand, when the heat-denatured DNA and C-RNA are maintained at "quenching" conditions, almost no DNA-C-RNA complex forms. In other words, the concentration of nucleic acids in this experiment is evidently not high enough to give nonspecific aggregates that are stable to gradient centrifugation (Fig. 4*b*).

The complex formation shown in Figure 4*a* is remarkable. At the concentration of RNA (approximately 100 γ/ml) and DNA (approximately 200 γ/ml) employed in the denaturation and annealing, DNA-RNA complex formation occurs to the complete exclusion of DNA renaturation. That is, in the competition between the

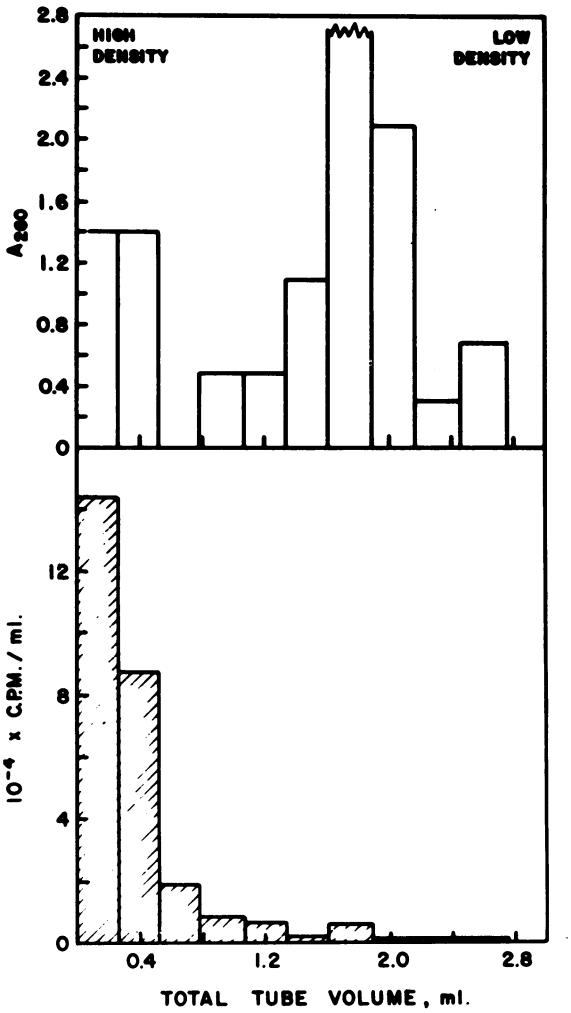

FIG. 2.—Distribution of P^{32}-labeled T2-C-RNA and template T2 DNA in a CsCl density gradient. The volume is calculated from the bottom of the centrifuge tube. For the absorbance measurements, fractions 1 and 2 were combined, and fraction 3 was not measured. *Preparation of RNA and DNA:* Method A (see *Materials and Methods*). *Centrifugation:* 35,000 rpm, 96 hr, near 0°C, 8.4 molal CsCl. *Analysis:* Each fraction is diluted to 0.8 ml and the absorbance measured at 260 mμ. An aliquot is then taken for measurement of TCA-insoluble radioactivity. The concentration of 260 mμ absorbing material and label in the original fraction is calculated from the known volumes, and shown as A_{260} and C.P.M./ml respectively.

two types of equilibria

$$\text{T2-C-RNA} + \text{heated T2-DNA} \rightleftarrows \text{DNA-C-RNA}$$

and $$\text{heated T2-DNA} + \text{heated T2-DNA} \rightleftarrows \text{DNA-DNA (helical)},$$

the former is greatly favored. Upon CsCl gradient centrifugation, the entire DNA band is vacated and a new, considerably denser, band due to DNA-C-RNA complexes appears.

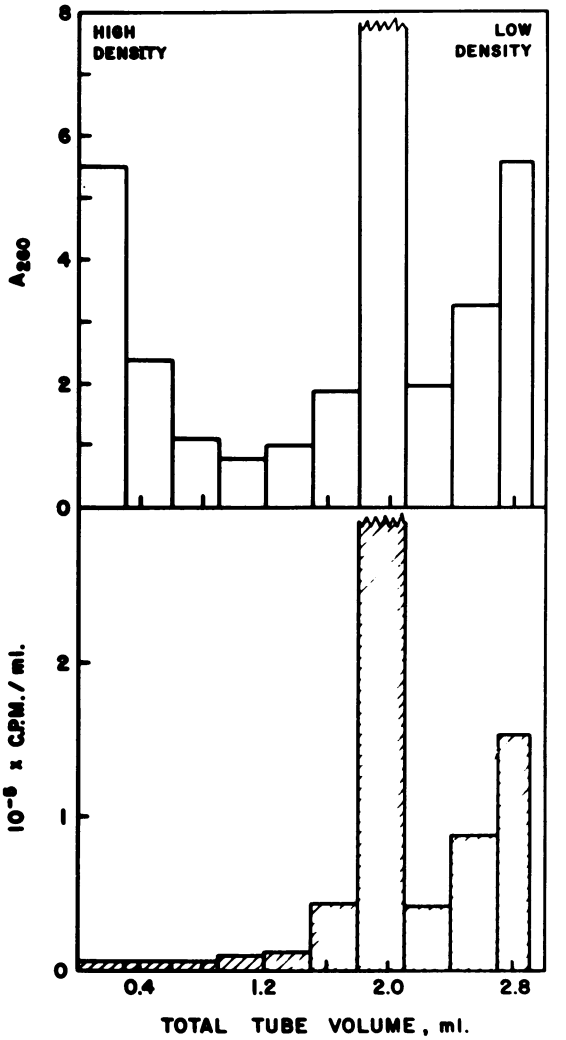

FIG. 3.—Distribution of unlabeled T2-C-RNA and template T2-DNA-H^3 in a CsCl density gradient. Preparation of nucleic acids, centrifugation, and analysis as described in Figure 2.

(3) *Is the DNA-C-RNA complex specific?* T2-C-RNA has been isolated by density gradient centrifugation and separately annealed with denatured T2 DNA (35 mole % GC), sea urchin DNA (Strongylocentrotus, 38 mole % GC), and *E. coli* DNA (51 mole % GC). In this experiment, both DNA (approximately 90 γ/ml) and RNA (*ca.* 10 γ/ml) were more dilute in the annealing mixture, so that complexation was less extensive than observed in the previous experiment. The results are shown in Figure 5. T2 and sea urchin DNA are very close in average base composition. Nevertheless, under conditions in which complex formation between T2 DNA and T2-C-RNA is clearly demonstrable, none occurs between sea urchin or *E. coli* DNA and T2-C-RNA. In fact, no interaction between T2-C-RNA and the heterologous sea urchin DNA occurs at the higher concentration of the

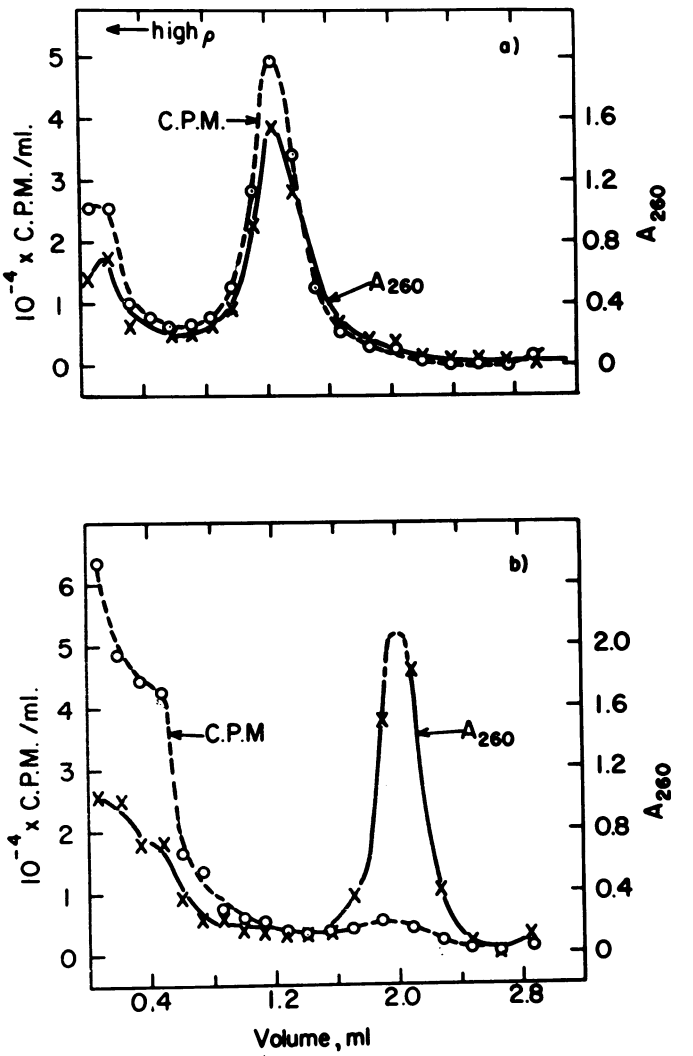

FIG. 4.—Conditions for the Formation of DNA-C-RNA Complexes.

(*a*) Distribution of a heated and annealed mixture of P^{32}-labeled T2-C-RNA and T2-DNA in a CsCl density gradient. ×: A_{260}. ○: C.P.M. *Preparation of RNA:* Method B. *Denaturation and annealing:* 0.07 μmoles T2-C-RNA-P and 0.1 μmoles T2-DNA-P in 0.22 ml 2 *M* CsCl are heated to 100°C for 10 min, quenched, and equilibrated 12 hr at 40.1°C. The mixture is then made up to 3 ml of 8.4 molal CsCl, 0.01 *M* Tris, pH 7. *Centrifugation:* 35,600 rpm, 75 hr, 25°C. *Analysis:* as in Figure 2.

(*b*) Distribution of a heated and quenched mixture of P^{32}-labeled T2-C-RNA and T2 DNA. ×: A_{260}. ○: C.P.M./ml. *Denaturation and quenching:* 0.07 μmoles T2-C-RNA-P and 0.1 μmoles T2-DNA-P in 0.22 ml 2 *M* CsCl, heated to 100°C for 10 min, quenched and maintained at 0°C for 12 hr. The mixture is then made up to 3 ml of 8.4 molal CsCl, 0.01 *M* Tris pH 7. *Centrifugation and analysis:* as in (*a*).

experiment discussed in the previous section, and shown in Figure 4 (data are not presented here).

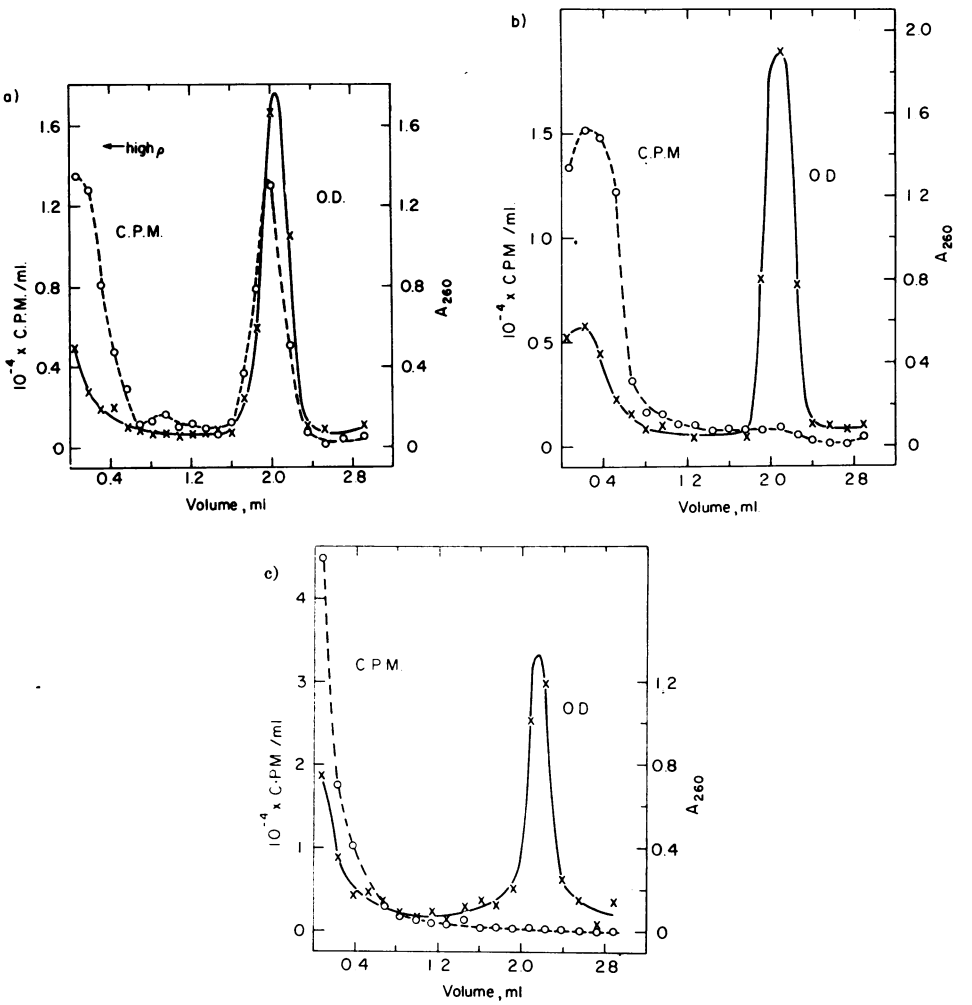

FIG. 5.—The specificity of DNA-C-RNA complexes. Distribution of heated and annealed mixtures of P^{32}-labeled T2-C-RNA with:

(*a*) T2-DNA (35 mole % GC).
(*b*) Sea urchin DNA (38 mole % GC).
(*c*) *E. coli* DNA (51 mole % GC) in a CsCl density gradient. ×: A_{260}. ○: C.P.M./ml. *Preparation of RNA:* Method B.

Denaturation and annealing: (*a*) 0.02 μmoles T2-C-RNA-P; 0.12 μmoles T2 DNA-P; (*b*) 0.02 μmoles T2-C-RNA-P 0.10 μmoles sea urchin DNA-P; (*c*) 0.02 μmoles T2-C-RNA-P 0.10 μmoles *E. coli* DNA-P; each in 0.35 ml of 0.6 *M* CsCl. Samples are heated in boiling water for 10 min, quenched, and equilibrated 10 hr at 37°C. Each mixture is then made up to 3 ml of 8.4 molal CsCl, 0.01 *M* Tris pH 7.

Centrifugation: 35,600 rpm; 77 hr; 25°C; 0.01 *M* Tris.

(4) *Is the DNA-C-RNA complex unique?* It appears from the experiments shown in Figures 4*a* and 5*a* that the density of DNA-C-RNA complexes depends on the experimental conditions of the denaturation and annealing and specifically, in these two experiments, on the concentrations of both components. When complex formation is incomplete, the DNA-C-RNA is only slightly heavier than DNA. When complex formation is very extensive, the density of DNA-C-RNA complex

(or complexes) is greatly increased. The evidence of these experiments and others not recorded here is that the composition of DNA-C-RNA may vary over a wide range. Undoubtedly this is, at least in part, due to the disparity between the molecular weights of T2 DNA, and the T2-C-RNA synthesized by the partially purified RNA polymerase.

(5) *Does RNA polymerase priming cause DNA denaturation?* Figure 6 shows a comparison of the buoyant densities of unreacted phage T2 DNA and of T2 DNA which has been used as template in a C-RNA synthesis. There appears to be no change of DNA buoyant density as a result of the RNA synthesis. In the light of these experiments, this result is capable of only two interpretations: (1) phage DNA is not irreversibly denatured by its participation as a C-RNA template, or (2) only a small number of the phage DNA molecules act as C-RNA template, and this experiment has failed to demonstrate the behavior of this small fraction. The

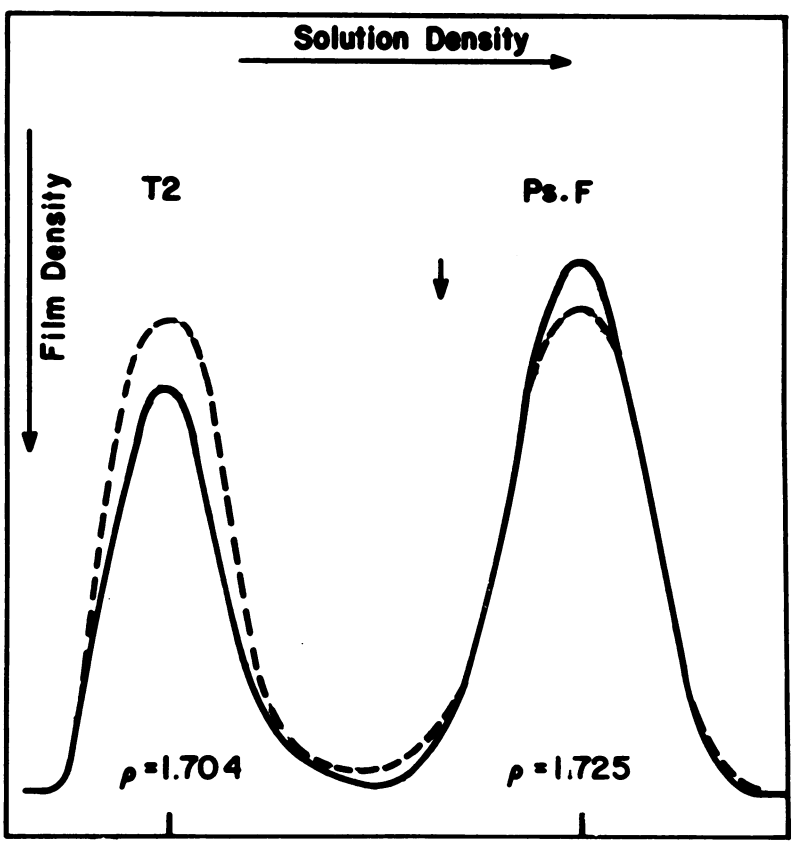

FIG. 6: The CsCl buoyant density of template T2 DNA and T2 DNA which has not taken part in RNA synthesis. *Ps. fluorescens* DNA is used as a density marker. Data are presented in terms of densitometric traces of absorption camera pictures through the region of the DNA "bands." The vertical arrow shows the anticipated location of denatured T2 DNA. The buoyant densities of T2 and *Pseudomonas* DNA are taken from published data.[30, 31]

Centrifugation: 7.7 molal CsCl, 0.01 *M* Tris pH 8, 44,700 rpm, 25°C, 24 hr. 0.60 ml solution are contained in a 12-mm Kel-F cell equipped with a 1° negative prismatic window.

second alternative is rejected on the grounds that the maximum quantity of T2-DNA having changed density (i.e., having been denatured) which could escape detection is not more than 10 % of the total. If, however, only 10 per cent of the molecules of the DNA preparation were active as primer, this could only have come about by 90% primer inactivation during isolation: the inactivation could not entail denaturation. This seems unreasonable, since all the T2 DNA preparations used by us have had comparable priming activities and compare favorably with DNA primers derived from other sources and isolated in many different ways. Furthermore, the RNA synthesis for this experiment was carried out at a DNA concentration lower than, and enzyme concentration close to, saturation. All of these considerations militate against the possibility that less than 10 per cent of the DNA molecules prime all of the C-RNA synthesis while the bulk of the DNA remains inactive.

Comments.—At this point, the relationship between C-RNA and its DNA template has been determined at several levels of structure. It has been shown previously that the average composition of C-RNA's and their DNA primers correspond, [1,21,22] and the preceding communication demonstrated that the nearest-neighbor frequencies of four different C-RNA's and their DNA primers also correspond.[2] These two experiments suggest, but by no means prove, that C-RNA is a faithful copy of its DNA template. There is a tendency for many of the nearest-neighbor frequencies of a DNA to follow patterns consistent with its *average* base composition, so that the differences between DNA's of comparable average nucleotide composition, while evident, are by no means distinctive (ref. 23, Tables VI, VII, and VIII). Only when longer nucleotide sequences are investigated, do species specificities become more clear-cut.[11, 24] In these experiments, therefore, the ability of template T2 DNA to form specific complexes with T2-C-RNA is a test of the presence of entire nucleotide *sequences* in DNA and C-RNA chains that are simultaneously capable of binding to each other in the face of the competing tendency of complementary nucleotide sequences on DNA strands to recombine.[11]

We have evidence also that every strand of a T2 DNA sample is capable of forming such complexes, since in one experiment (Fig. 4*a*) we entirely eliminated material banding at the density of DNA. The simplest interpretation of this finding is that C-RNA molecules exist which are complementary to both strands of T2 DNA and that both DNA strands act as template, either separately or together.[25]

We turn next to a consideration of the manner in which the DNA is utilized in the C-RNA synthesis. Others have shown, as have we, that a double helix in which only a small portion of the nucleotide sequence is maintained in register at all times can rewind in such a manner as to obliterate, for the purposes of the density-gradient experiment, all traces of its unfolding.[26–28] In order to account for the unchanged buoyant density of template DNA (Fig. 6), we must therefore assume that DNA double helices do not have to unwind entirely in serving as RNA templates. In addition, we know from the annealing experiments (Fig. 4*a*) that DNA-C-RNA complexes involving entire C-RNA molecules must be very stable. The clear separation of T2-C-RNA from its template argues against any C-RNA synthetic mechanism involving such intermediates. We are, therefore, left with two alternatives: (1) The template for RNA synthesis is a single polynucleotide

strand of DNA, of which only the small region immediately surrounding the site of terminal nucleotide addition is unwound, or (2) the double helix may act as a template without unwinding.

The similarity of the RNA and DNA syntheses on a DNA template is striking. However, in this study a difference of great importance can be observed. While the *in vitro* DNA template synthesis involves recombination of template and product polynucleotide,[29] the *in vitro* synthesized C-RNA is free of its template. Finally, let us suppose that the mechanism of the *in vivo* formation of messenger RNA is the same as that of C-RNA *in vitro*. In that case, it seems probable that as the assembly of the RNA is completed, the two nucleic acids part and the DNA remains in, or spontaneously returns to, its double helical form.

We should like to express our thanks to S. Spiegelman for his generous advice, to L. M. Kozloff and R. Mackal for gifts of bacteriophage, to J. Moohr for preparing tritium-labeled phage, and to G. Gibson for constructing a fraction collecting device.

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† Operated by the University of Chicago for the U.S. Atomic Energy Commission.

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[2] *Ibid.*, **47**, 1400 (1961).

[3] A number of different enzymes (polynucleotide phosphorylase, RNA polymerase, polyribonucleotide synthetase) from various sources have now been reported for the synthesis of RNA.[4-6] Each of these reactions has different properties, and the products formed, although exhibiting characteristic polynucleotide linkages, are not the same. Furthermore, in recent years, a distinction between RNA molecules has been made by various investigators based upon their cellular origin and biological properties, e.g., soluble RNA,[7] amino acyl RNA,[8] and their chemical composition, e.g., "messenger" RNA.[9, 10] In view of this, we have tentatively assigned the name of "complementary RNA" to the product of the reaction catalyzed by RNA polymerase, in order to designate a specific type of RNA, that is, one which is formed from a DNA template, contains a similar base composition and nearest-neighbor frequency to that of the primer, and exhibits the type of complementary properties with its primer first described by Hall and Spiegelman.[11]

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[17] Abbreviations: C-RNA, complementary (biosynthetic) RNA; T2-C-RNA, complementary RNA synthesized on a bacteriophage T2 DNA template; DNA-C-RNA, a complex between DNA and complementary RNA; CTAB, cetyltrimethylammonium bromide; TCA, trichloracetic acid; G, guanine; C, cytosine; Tris, tris-(hydroxymethyl)-aminomethane; A_{260}, absorbance at 260 mμ; s, svedberg unit.

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[25] One other, less plausible possibility exists which, however, cannot be excluded. It is that the DNA-C-RNA complexes of Figure 4*a* consist of networks, each involving many DNA and RNA chains. In such a network, DNA-C-RNA complexes might involve one type of DNA strand *only*, while the complementary DNA strand is carried along solely because of its ability to form intermittent DNA-DNA complexes.
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*STUDIES ON THE KINETICS OF PROTEIN SYNTHESIS IN YEAST**

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It is now generally agreed that ribosomal particles are the primary site of protein synthesis. In yeast, this evidence is supported by studies on the incorporation of amino acids *in vivo*[1, 2] and *in vitro*[3] and by the finding on ribosomes of bound protein[4] and enzymes[5] which are presumably newly synthesized protein. One question raised by these findings is what percentage of the ribosomal protein is represented by newly synthesized polypeptides and what is the relationship between this material and the structural proteins of the ribosome? In an attempt to further characterize the nascent ribosomal protein, this material was isolated and compared with the structural protein of the ribosomes. These two classes of protein differed not only in their composition and properties but also in the kinetics of synthesis. The nascent protein has a transient existence on the ribosome and the kinetics of its turnover is sufficient to account for the over-all rate of protein synthesis.

Methods.—A diploid yeast (*Saccharomyces dobzanskii* × *Saccharomyces fragilis*) was used in these experiments. Unless otherwise specified, all cultures were grown in a sulfur-low, synthetic medium prepared by adding to one liter of water: NH_4Cl, 6.4 gm; K_2HPO_4, 17.4 gm; succinic acid, 11.6 gm; $CaCl_2$, 0.2 gm; $MgCl_2$, 0.2 gm; $(NH_4)_2SO_4$, 5 mg; sulfur-free trace elements; and vitamin mixture. The cultures were grown at 30° in shaken Erlenmeyer flasks. Under the conditions employed, the generation time was about 190 min.

For the kinetic studies on S^{35} and C^{14} amino acid incorporation, yeast cells were grown in the above medium until the optical density had reached a level of about