*A COMPLEX OF ENZYMATICALLY SYNTHESIZED RNA AND TEMPLATE DNA**

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If a reaction mixture containing DNA as template, DNA-dependent RNA polymerase, and the ribonucleotide-5′-triphosphates of adenine, guanine, uracil, and cytosine (ATP, GTP, UTP, and CTP) is incubated, the nucleotides are polymerized to RNA.[1–5] Since the synthesized RNA has the same nearest neighbor base relations as the template DNA,[6, 7] and—if the latter is double-stranded DNA—stimulates amino acid incorporation in an *in vitro* system for protein synthesis,[8, 9] it may be assumed that this *in vitro* synthesis of RNA on a DNA template is a model for the *in vivo* synthesis of messenger RNA. In order to understand how a single-stranded RNA molecule is made on a double-stranded DNA template, it would be desirable to know the structure of the intermediate reaction product, that is, of the complex formed by a nascent RNA molecule and its template DNA. One step in this direction is the identification of the complex and its separation from the other constituents of the reaction mixture.

In previous experiments[7, 10] in which the reaction mixture was deproteinized and then analyzed on a cesium chloride density gradient, no DNA-RNA "hybrids," similar to those formed upon heating and slow cooling of DNA with its homologous RNA,[11, 12] were found. However, the complex need not resemble such hybrids, but may, instead, be held together by the polymerase at the point of growth of the nascent RNA molecule.[10] As the experiments to be reported here show, the complex can, in fact, be isolated if the reaction mixture is subjected without deproteinization to zone sedimentation in a sucrose gradient. This method also allows study of the growth of the RNA molecules during the reaction and thus provides an insight into the molecular kinetics of the RNA polymerization.

Materials and Methods.—RNA polymerase was prepared according to the method of Chamberlin and Berg[5] except that DEAE Sephadex (Pharmacia, Upsala, Sweden, Type A 50, med.) instead of DEAE cellulose was used. The column was equilibrated with 0.005 *M* imidazole-HCl buffer, pH 7, containing 10^{-2} *M* $MgCl_2$, 10^{-4} *M* EDTA, 10^{-2} *M* β-mercaptoethanol. The partially purified enzyme preparation was dialyzed for 2 hr against the same buffer before it was adsorbed to the column. It was eluted with a gradient of 0.3–0.5 *M* KCl in the above buffer. The bulk of the polymerase activity was eluted between 0.35 and 0.4 *M* KCl; the specific activity was 2,500 units/mg using the same definition of the unit of activity as Chamberlin and Berg.[5] The polymerase was kept frozen in liquid nitrogen. After thawing 30–60% loss of activity was observed. The amount of protein in the polymerase preparation was determined from its optical density at 280 mμ assuming that 1 OD unit corresponds to a protein concentration of 1 mg/ml.

P^{32}-labeled T4 DNA was prepared by phenol extraction (2 × phenol, 3 × ether) of a T4 phage stock grown on *E. coli* B/r in $P^{32}O_4^{---}$ containing medium. This DNA contained 0.2 P^{32} atoms per unit of T4 DNA, and its sedimentation constant of 54S corresponded to that of whole T4 molecules.[13] The sedimentation profile of this high molecular weight DNA is strongly dependent on the DNA concentration, in agreement with the results of Burgi and Hershey.[13]

The DNase activity of the polymerase parparation was determined by incubating for 10 min at 37°C 50 μg/ml of P^{32}-labeled T4 DNA with various concentrations of polymerase in the same buffer as used for the RNA polymerization and then subjecting the DNA to zone sedimentation analysis on sucrose gradients. For 2, 10, or 50 μg/ml of polymerase the average sedimentation

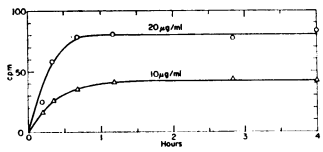

FIG. 1.—Kinetics of RNA synthesis with DNA-dependent RNA polymerase. A reaction mixture, containing 50 μg/ml T4 DNA, 270 mμmoles/ml of each ATP, GTP, UTP, C^{14}-labeled CTP (150 cpm/mμmole), and 10 or 20 μg/ml polymerase (specific activity 1400 units/mg), respectively, all made up in buffer (see *Materials and Methods*), was incubated at 37°C; 0.1-ml samples were precipitated with 200 μg of carrier DNA in 5 ml cold 0.5 *M* TCA. The precipitates were washed and dried on Millipore filters, and their radioactivity determined on a gas-flow counter.

velocity of the DNA was found to be 45S, 28S, and 13S, respectively. Using the equation of Burgi and Hershey,[13] which correlates the sedimentation velocity of the DNA with its molecular weight ($S_{20} = 0.080\ M^{0.35}$) it was calculated that under these conditions on the average 1, 8, or 70 complete cleavages, respectively, were produced per whole T4 DNA molecule (molecular weight 1.3×10^8).

The buffer used for enzyme dilutions and reaction mixtures contained 0.05 *M* Tris-HCl pH 7.9, 10^{-3} *M* $MnCl_2$, 2×10^{-3} *M* $MgCl_2$, 10^{-2} *M* β-mercaptoethanol.

Results.—Kinetics of the RNA polymerization reaction: Under our conditions the rate of RNA synthesis decreases after about 20 min, and is essentially zero after 100 min (Fig. 1). Addition of more DNA or triphosphates at 100 min did not cause RNA synthesis to resume. However, if fresh polymerase was added to the reaction mixture at 100 min, RNA synthesis resumed. This suggests that the cessation of RNA synthesis observed is due to inactivation of the polymerase itself.

It can be seen that both the initial rate of synthesis and the final level of RNA are proportional to the polymerase concentration, at least for the two concentrations shown. If the molecular weight of the RNA made at the two enzyme concentrations were the same, then there appears to be a constant ratio between the number of RNA molecules made and the number of polymerase molecules introduced. That this constant ratio exists, and that it is equal to 1, will be shown in the following sections.

The complex between RNA and DNA: In order to demonstrate a complex between newly made RNA and DNA template molecules, a reaction mixture containing P^{32}-labeled T4 bacteriophage DNA, DNA-dependent RNA polymerase, ATP, GTP, UTP, and H^3-labeled CTP was incubated for 10 min at 37° C, and the polymerization reaction was stopped by 10-fold dilution into chilled buffer. One sample of this diluted mixture was layered on a sucrose gradient and subjected to zone sedimentation analysis (Fig. 2*a*). The DNA was found to have an average sedimentation constant of approximately 40S, indicating that it had been slightly degraded during incubation (see *Methods*). The distribution of the RNA has a maximum which coincides with that of the DNA distribution. (The H^3 activity near the meniscus, which corresponds to 0.1 per cent of the unincorporated triphosphates, is due to contamination by triphosphates and is not RNA.) This suggests that *the newly made RNA formed by the polymerization reaction is bound to its DNA template.*

To a second sample of the diluted reaction mixture, the protein denaturing agent sodium dodecyl sulfate (SDS) was added to a final concentration of 0.4 per cent, and the sample was then analyzed as before (Fig. 2*b*). This treatment with SDS has produced an important change in the sedimentation profile: whereas the template DNA still has an average sedimentation constant of 40S, the RNA now moves more slowly, having an *average* sedimentation constant of only 12S. That is, *treatment*

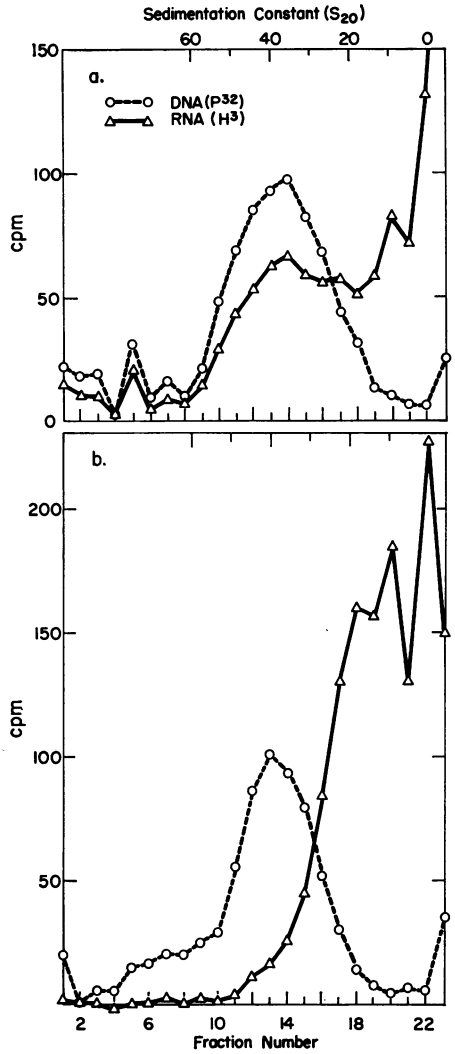

FIG. 2.—Sedimentation distribution of DNA and RNA from a DNA-dependent RNA polymerization. (*a*) Untreated sample of the reaction mixture; (*b*) SDS-treated sample of the same reaction mixture (held 3 min at 37° C in 0.4% SDS). The reaction mixture containing 50 μg/ml of P^{32}-labeled T4 DNA, 200 mμmole/ml of each ATP, GTP, UTP, H^3-labeled CTP (3×10^4 cpm/mμmole) and 2 μg/ml of polymerase, all made up in buffer (see *Materials and Methods*), was incubated for 10 min at 37°C. Samples of 0.1 ml were diluted into 0.9 ml of chilled buffer or 0.4% solution of SDS, respectively, layered onto 25 ml of a 4–20% sucrose gradient made up in the same buffer (except for the SDS-treated sample: 0.1 *M* NaCl, 0.01 *M* tris pH 7.5), and centrifuged 5.5 hr at 25,000 rpm in an SW 25 Spinco swinging bucket rotor at approximately 4°C. Fractions of 10 drops each were collected through a hole punched into the bottom of the centrifuge tube; precipitation of DNA and RNA in the fractions was effected by 5 ml of 0.5 *M* TCA with 200 μg carrier DNA. The precipitates were collected, washed, and dried on Millipore filters, and their radioactivity was assayed in a liquid scintillation counter. About 0.1% of the free radioactive CTP in the fractions was observed to stick to the filters. This background activity is found in the last fractions near the meniscus.

with SDS has freed the newly made RNA from its DNA template, since the two polynucleotide species now sediment independently.

If only nascent RNA molecules were bound to DNA, while those completed come free, and if the time needed to complete the RNA molecules were short compared to the time the reaction mixture was allowed to incubate, then two classes of RNA molecules should be seen: those bound to the DNA and those which have come free. To search for this free RNA, 3 aliquots were taken from a reaction mixture after 5, 10, and 20 min of incubation, diluted, chilled, and analyzed by zone sedimentation (Fig. 3). Here it is seen that there is no demonstrable free RNA (unless it has a small sedimentation constant and is obscured by the unincorporated triphosphates near the meniscus). Instead, the amount of RNA sedimentating with the DNA steadily increases with time. In another experiment, shown in Figure 4, the reaction mixture was incubated for 60 min and analyzed as before except that the

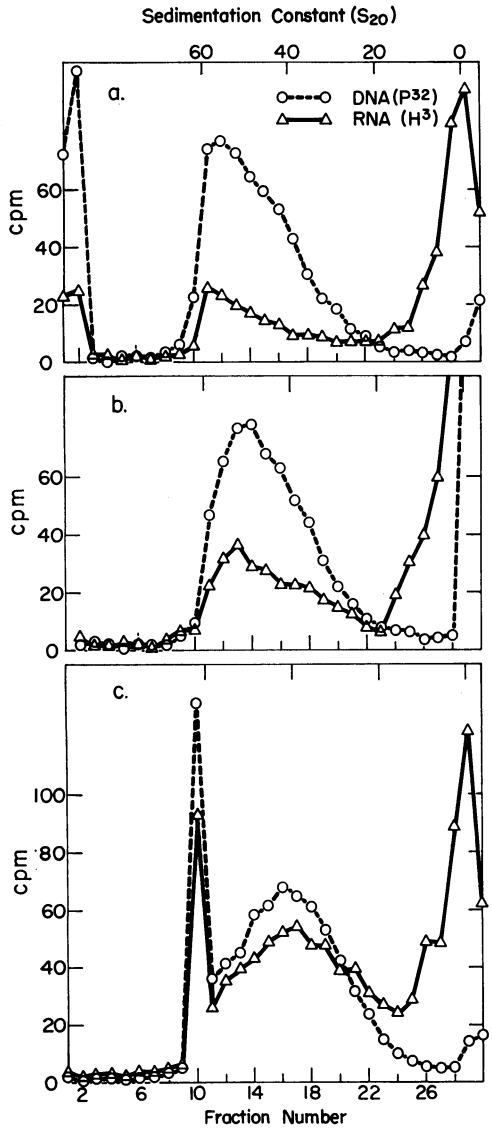

FIG. 3.—Zone sedimentation analysis of a reaction mixture incubated at 37°C for (*a*) 5 min, (*b*) 10 min, (*c*) 20 min. Experiment as in Fig. 2*a* except that samples were layered onto 29 ml of a 4–20% sucrose gradient and centrifuged for 12 hr at 22,000 rpm.

fractions of the sucrose gradient near the meniscus were assayed for radioactivity after double precipitation to lower the contamination from unincorporated triphosphates. Here the coincidence of the RNA and DNA distributions is even more striking than in Figure 3. There is, therefore, no demonstrable free RNA in any of the reaction mixtures; instead, most of the RNA remains bound to the DNA throughout the course of the reaction. However, it should be noted that the amount of free RNA may be as much as 20 per cent of the total without being evident in the sedimentation distribution. To see whether the RNA attached to DNA seen in Figures 2*a*, 3*a*,*b*,*c*, and 4 is attached only to template DNA or whether a complex can be formed with nontemplate DNA and therefore be of no functional significance, P^{32}-labeled T4 DNA was broken by hydrodynamic shear into small molecules (= small DNA) easily distinguishable in zone sedimentation from the large molecules used as templates in previous experiments (= large DNA). In a first reaction mixture large DNA was used as a template at the start of incubation, and 6 min later an equal amount of small DNA was added and incubation continued an additional 6 min. In a second reaction mixture the small DNA was added first, and the large DNA added for the second period. Each mixture was diluted and analyzed as before. The result (Fig. 5) was that in both reaction mixtures *all the RNA is bound only to the DNA species initially present.* Thus, the binding between RNA and DNA occurs early in the reaction and is irreversible.

Molecular kinetics of the polymerization: In the preceding section it was shown that the RNA synthesized by the polymerase is bound to the DNA irreversibly, and that the choice of template occurs early in the reaction. This suggests the possibility that all the RNA molecules start growing at the outset and then continue to grow throughout the reaction. If this were true, the average molecular weight

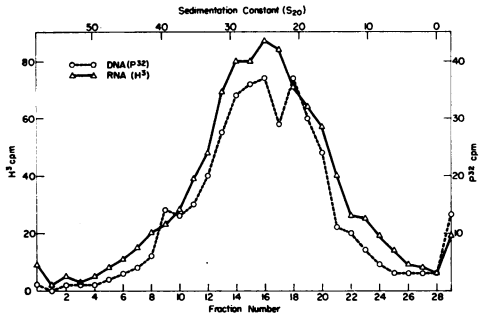

FIG. 4.—Zone sedimentation analysis of a reaction mixture incubated for 60 min at 37°C. Experiment as in Fig. 2*a* except that samples were layered onto 29 ml of a 4–20% sucrose gradient and centrifuged for 16.6 hr at 25,000 rpm. The nucleic acids in the last 7 fractions near the meniscus have been double-precipitated to reduce the background activity caused by free radioactive CTP.

and therefore the sedimentation constant of the RNA in the reaction mixture should increase with time. That this is true can be seen from the sedimentation profiles in Figure 6*a* of three aliquots of a reaction mixture, taken after 5.8, 10, and 20 min of incubation and treated with SDS to free the RNA from the DNA. (The sedimentation properties of this RNA did not change when deproteinized with phenol, and for simplicity deproteinization with phenol was usually omitted.) In Figure 6*b*, the square of the observed sedimentation constant of the RNA product (a quantity roughly proportional to the molecular weight of RNA with a random coil structure[14]) has been plotted against the total amount of RNA formed at six different reaction stages, in which the sedimentation constant ranged from 9S (m.w. 1.5×10^5) at 3 min, to 32S (m.w. 2×10^6) at 60 min. The constant ratio of molecular weight to total weight seen here suggests that a constant number of RNA molecules exist throughout the course of the reaction.

This conclusion would be strengthened if it were shown that *all* RNA molecules grow. In order to demonstrate this, an experiment was carried out in which the polymerization mixture contained initially a low concentration of H^3-CTP of high specific radioactivity. After the reaction had been allowed to progress for a short time, further incorporation of the labeled nucleotide was effectively stopped by addition of an excess of nonlabeled CTP. One sample of the mixture was removed at this point and the reaction allowed to proceed for a longer time, after which a second sample was removed. The sedimentation profile of the RNA in both samples was then examined after treatment with SDS. The results of this experiment are presented in Figure 7, where it may be seen that the RNA formed after a short

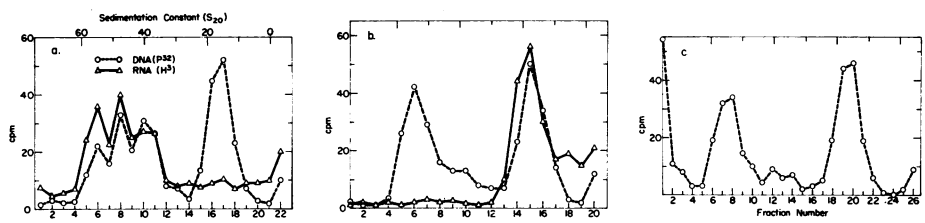

FIG. 5.—DNA-RNA complex formation before and after 6 min of incubation. (*a*) 0.2 ml of a reaction mixture as described in Fig. 2 was incubated at 37°C for 6 min, at which time 0.05 ml of a 200 μg/ml solution of low molecular weight DNA was added and the incubation continued an additional 6 min. A 0.1-ml aliquot was then diluted into 0.9 ml of chilled buffer. (*b*) A reaction was carried out as in (*a*) except that the order in which the two kinds of DNA were added was reversed. (*c*) A mixture of the two kinds of DNA used in (*a*) and (*b*) was made in buffer, at the same concentrations as present in the reaction mixtures after dilution. The three above samples were subjected to zone sedimentation for 9.6 hr at 25,000 rpm. The last 8 fractions near the meniscus have been double-precipitated to reduce the background activity. The low molecular weight DNA was prepared by repeatedly forcing a 200 μg/ml solution of P^{32}-labeled T4 DNA through a 27-gauge hypodermic needle at a rate of about 1 ml/sec.

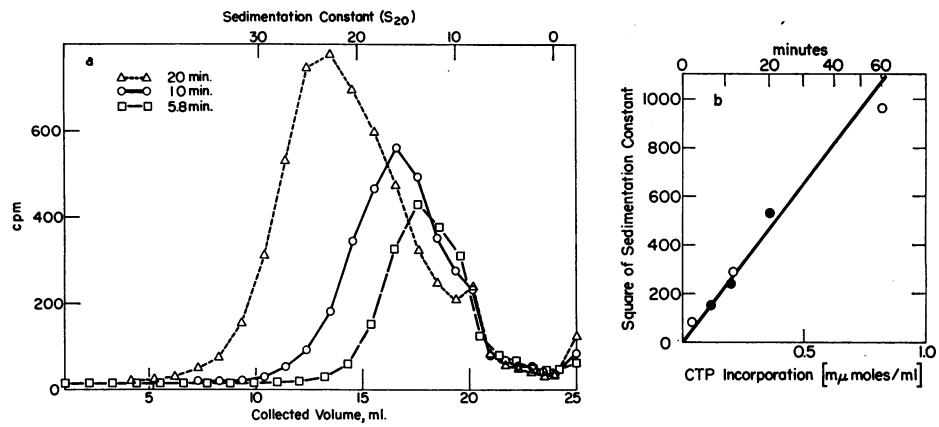

FIG. 6.—(*a*) Zone sedimentation analysis of RNA obtained from a reaction mixture incubated at 37°C; samples taken at 5.8, 10, and 20 min, and treated with SDS. Experiment as in Fig. 2*b*, except that samples were centrifuged 12.1 hr at 25,000 rpm. (*b*) The square of the sedimentation constants obtained from Fig. 6*a* (●) and three other experiments (○), plotted against CTP incorporation.

reaction time sediments with a velocity of about 6S, and that after further progress of the reaction nearly all of the H^3-label incorporated into small RNA molecules at early stages of the reaction has become part of large RNA molecules that sediment at the much higher velocity of 16S. (The overlap at the trailing ends of the two sedimentation bands is due to the contaminating presence of unconverted labeled substrate.) These results, therefore, show that most RNA molecules which started early continued to grow throughout the incubation.

Discussion.—(1) *Significance of the complex:* The complex observed in this study is likely to be functionally significant and involve the growing point of the RNA molecules, since it is only formed early in the reaction when all RNA chains are initiated.

(2) *Absence of spontaneous dissociation of the DNA-RNA complex:* This, at first surprising, result is in fact to be expected, at least in the early part of the reaction, if all RNA molecules are growing. The situation may be different at the end of the reaction, when the decreasing rate of polymerization may either reflect a lower growth rate of all individual polynucleotide chains until their growth finally stops altogether, or mean that growth of more and more RNA molecules stops abruptly until finally growth of all molecules has stopped.

It may be asked if an *in vitro* system in which DNA acts as a template for RNA synthesis, but in which the finished RNA does not seem to come free of the DNA, is a useful model for the synthesis of messenger RNA *in vivo*, where it is known that the messenger RNA comes free of the DNA. It may be proposed that *in vivo* an active process, perhaps involving the ribosomes, frees the newly made messenger RNA molecule from its template. Thus in the system studied here, the liberation of completed RNA molecules could not take place.

(3) *Dissociation of the complex into free DNA and free RNA by detergent:* Since detergents such as SDS denature proteins, it is concluded that the nascent RNA is held by a protein to its template DNA. It is then plausible to assume that this protein is the polymerase.

If it is true that the polymerase serves to bind RNA and DNA in the complex, a structure for the complex may be proposed, in which the polymerase molecule and a few of the most recently added nucleotides constitute the region of contact between the DNA template and the growing RNA molecule. An alternate structure in which the whole length of the RNA molecule is in contact with the DNA is also possible, if the structure is stabilized at the point of RNA growth by the polymerase, and the structure becomes unstable as soon as the polymerase is removed or denatured.

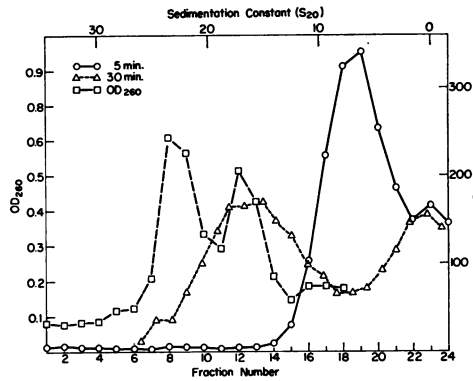

FIG. 7.—Fate of early synthesized RNA. A reaction mixture, as in Fig. 2, except containing only 0.8 mμmole/ml H^3-labeled CTP (3.5×10^5 cpm/mμmole), was incubated at 30°C for 5 min; then unlabeled CTP was added to a final concentration of 300 mμmole/ml. A sample of 0.6 ml was taken at this time, and after 25 min. Each sample was treated with SDS, analyzed, as described for Fig. 2*b*, and diluted to 1.0 ml with H_2O. 160 μg of ribosomal RNA (kindly supplied to us by C. G. Kurland) were added as a sedimentation marker, and the samples were layered on 24 ml of a sucrose gradient 0.1 *M* in NaCl and 0.01 *M* in Tris and centrifuged 15.3 hr at 25,000 rpm.

(4) *Slow growth of RNA molecules:* At 37°C RNA with a sedimentation constant of 23S was produced in about 20 min. Assuming that this RNA has a random coil structure and thus a molecular weight of 1.1×10^6, as it is reported for 23S ribosomal RNA from *E. coli*,[14] the growth rate of each RNA molecule would be 2.5 nucleotides per second. Assuming that in T4 bacteriophage-infected bacteria the first phage-induced enzymes are formed 2–4 min after infection[15] and assuming further that the messenger RNA molecules for these enzymes have a molecular weight of 5×10^5, the rate for the *in vivo* synthesis of messenger RNA would be at least 6–12 nucleotides/second which is at least twice as fast as the *in vitro* rate. The possibility that the polymerase used in our experiments was "slow" due to its partial inactivation seems unlikely since experiments made with polymerase preparations of different specific activities gave the same growth rate of individual RNA molecules, but gave different numbers of RNA molecules synthesized.

(5) *Constant number of RNA molecules in the reaction mixture:* This number can be calculated from the data in Figure 6*b*. 0.41 mμmole CTP per ml of reaction mixture was converted into RNA when the RNA had an average sedimentation constant of 23S ($S^2 = 529$). If this RNA has a molecular weight of 1.1×10^6 and contains 17 per cent cytosine, the number of RNA molecules per ml is 5×10^{11}.

The number of RNA molecules per DNA molecule: The DNA concentration used in our experiments (50 μg/ml) corresponds to 2.3×10^{11} T4 DNA molecules of a molecular weight of 1.3×10^8 per ml. With 5×10^{11}RNA molecules synthesized per ml reaction mixture, on the average two RNA molecules are started per whole T4 DNA template. Other experiments involving a higher concentration of polymerase or a lower concentration of DNA indicate that at least 50 RNA molecules can grow simultaneously on one T4 DNA molecule.

Estimation of the molecular weight of the polymerase: Since our results indicate that every active polymerase molecule in the reaction mixture synthesizes only one

RNA molecule, a maximum for the molecular weight of the polymerase may be calculated from the number of RNA molecules synthesized per weight of polymerase. If the pure enzyme has a specific activity 6,100 units/mg,[5] the molecular weight would be about 6×10^5.

Summary.—The *in vitro* synthesis of RNA catalyzed by purified DNA-dependent RNA polymerase from *E. coli* has been studied for the purpose of finding the complex between nascent RNA and its template DNA. The results of this study can be summarized as follows: (1) The complex can be isolated as a stable structure, consisting of DNA, RNA, and the polymerase, by zone sedimentation. (2) Most of the RNA remains bound to the DNA even at the end of the reaction. (3) The complex can be dissociated into free DNA and free RNA by detergent. (4) The RNA molecules grow slowly, at a rate of 2–3 nucleotides per second to a final molecular weight of 2×10^6. (5) The number of nascent RNA molecules is constant through the reaction: the synthesis of all RNA molecules starts during the first minutes of incubation, and during the rest of the reaction these molecules grow in size.

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