Template-Induced Dissociation of Ribonucleic Acid Polymerase*

David A. Smith, Aaron M. Martinez, Robert L. Ratliff, D. Lloyd Williams, and F. Newton Hayes

ABSTRACT: The sedimentation properties of ribonucleic acid (RNA) polymerase–polydeoxynucleotide complexes have been examined by analytical and density gradient centrifugation. Whereas free RNA polymerase from *Escherichia coli* had a sedimentation coefficient of about 24 S under the conditions utilized, when the enzyme was complexed with short polydeoxynucleotides its sedimentation coefficient was decreased. When the polynucleotide to enzyme mole ratio was about 0.4, a peak sedimenting at 19 S appeared in addition to the 24S peak. As the ratio was increased, the 24S boundary disappeared with concomitant formation of a boundary sedimenting at 13 S. When high ratios (*ca.* 18) were utilized, nearly all of the protein sedimented at 13 S. Addition of deoxyribonuclease caused a reversal to high molecular weight forms of the enzyme.

Analysis after density gradient centrifugation revealed that the 19S and 13S forms both contained bound polydeoxynucleotides.

The central role of RNA polymerase in the transfer of biological information stimulates interest in the structure and catalytic mechanism of this enzyme. We have begun studies aimed to help provide such information for RNA polymerase as isolated from *Escherichia coli* (Smith *et al.*, 1966, 1967). It is now generally agreed that RNA polymerase freshly isolated from *E. coli* is a large enzyme which has a sedimentation coefficient of 21–24 S in low ionic strength buffers. It undergoes complex dissociation–reassociation reactions upon raising the ionic strength or pH, or upon aging (Fuchs *et al.*, 1964; Colvill *et al.*, 1966; Richardson, 1966a; Stevens *et al.*, 1966; Ratliff *et al.*, 1967). Thus the question of the size of the active enzyme molecule is raised. Fuchs *et al.* (1964) suggested that the 24S form of the enzyme was the "native" form. Richardson (1966a,b) also favored the large form, since he found that under ionic conditions which favor dissociation no catalytic activity could be detected. However, our experiments indicated that under these ionic conditions RNA polymerase activity might or might not be demonstrable, depending upon the particular template in use (Ratliff *et al.*, 1967). In order to obtain more direct evidence regarding the size of the active RNA polymerase molecule, we have investigated the sedimentation properties of complexes of the enzyme and small polynucleotides.

Materials and Methods

Materials. RNA polymerase was isolated as previously described (Ratliff *et al.*, 1967) and stored under liquid nitrogen. The specific activity of the preparations used in these experiments was about 2500 units/mg. Deoxythymidylate oligomers were synthesized by the method of Khorana and Vizsolyi (1961), and deoxycytidylate oligomers were synthesized by the method of Khorana *et al.* (1961). These oligodeoxynucleotides were enzymatically lengthened through the use of terminal deoxynucleotidyl transferase, and the products were isolated as previously described (Bollum *et al.*, 1964; Hayes *et al.*, 1966). The average number of monomer units in the enzymatically lengthened polydeoxynucleotides was estimated from the reaction conditions utilized during their synthesis and from their sedimentation properties. The lengths of these polymers follow a Poisson distribution (Hayes *et al.*, 1967; Kato *et al.*, 1967). Extinction coefficients used in determining polydeoxynucleotide concentrations were for polydeoxycytidylate $\epsilon(P) = 7.6 \times 10^3$ and for polydeoxythymidylate $\epsilon(P) = 8.3 \times 10^3$ (pH 7, λ_{max}). tRNA was isolated from *E. coli* by the method of Zubay (1962). Radioactive nucleotides were obtained from Schwarz BioResearch. Unlabeled nucleotides were obtained from Calbiochem and P-L Biochemicals. Deoxyribonuclease (DNase I) was obtained from Worthington Biochemical Corp. Micrococcal DNase was a product of Miles Chemical Co. Other materials were obtained from standard commercial sources.

Analytical Methods. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Polynucleotide concentrations were determined spectrophotometrically. RNA polymerase was assayed by following the conversion of a radioactive nucleoside triphosphate into an acid-insoluble form as described previously (Ratliff *et al.*, 1967).

Density Gradient Centrifugation. RNA polymerase and polymerase–polynucleotide mixtures in a volume of 0.1 ml were layered over linear gradients of 5–15% glycerol. The gradients also contained 10 mM NaCl,

* From the Biomedical Research Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico. *Received July 19, 1967.* This work was performed under the auspices of the U. S. Atomic Energy Commission.

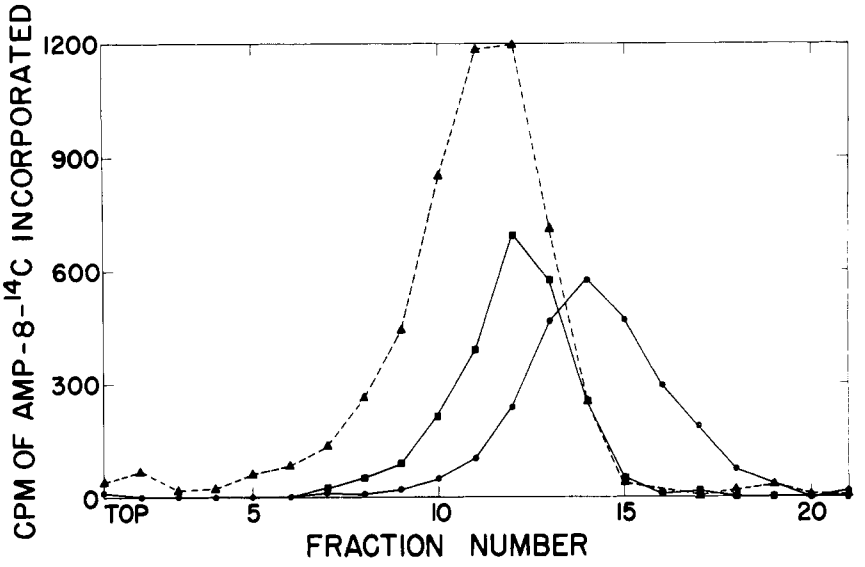

FIGURE 1: Sedimentation of RNA polymerase and RNA polymerase-d(pT)$_{13}$ mixtures in 20–35% glycerol gradients. RNA polymerase and polymerase-d(pT)$_{13}$ mixtures were layered over gradients and centrifuged as described under Materials and Methods. Fractions were assayed using salmon sperm DNA as template. The labeled nucleoside triphosphate was ATP-8-^{14}C (2.9 × 10^5 cpm/μmole). RNA polymerase (94 μg) alone (●—●); RNA polymerase (94 μg) and 0.13 nmole of d(pT)$_{13}$ (■—■); and RNA polymerase (94 μg) and 0.78 nmole of d(pT)$_{13}$ (▲—▲).

50 mM Tris-HCl (pH 7.5), 1.0 mM EDTA, and 10 mM 2-mercaptoethanol. In some experiments, 60 μg of catalase ($s_{20,w}$ = 11.3 S) was added to serve as a standard. The mixtures were centrifuged in a SW-39 rotor at 38,000 rpm for 4.5 hr at 4°. In a few cases, 20–35% glycerol gradients were used and centrifuged 12 hr at 35,000 rpm. After centrifugation the tubes were punctured, and fractions were collected. RNA polymerase activity was assayed by two procedures. In experiments in which the enzyme was sedimented alone or with tRNA, the fractions were assayed as previously described (Ratliff *et al.*, 1967) with the addition of salmon sperm DNA, the four ribonucleoside triphosphates (one of which was labeled), and $MgCl_2$ and $MnCl_2$. The usual procedure, when the enzyme was sedimented with polydeoxynucleotide homopolymers, was to assay the fractions by the above method but also in reaction mixtures in which DNA was omitted and the only nucleoside triphosphate added was the one complementary to the homopolymer. The second method of assay, therefore, was a measure of enzyme which has template bound to it. The recovery of polymerase activity from the gradients as determined by the salmon sperm DNA assay was generally about 65%.

Ultracentrifugation. Sedimentation measurements were carried out in a Spinco Model E ultracentrifuge equipped with schlieren optics, phase plate, and rotor temperature indicator and control. Cells with Kel-F, 4° sector centerpieces, and a 12-mm light path were used. All ultracentrifuge runs were done at a rotor speed of 44,770 rpm at a temperature of 20°. Photographs were taken using Kodak metallographic plates, and the positions of the schlieren peaks were read directly with the use of a Gaertner microcomparator. Sedimentation coefficients were calculated from plots of log distance *vs.* time. The partial specific volume of RNA polymerase as calculated from the amino acid composition was 0.73 (Ratliff *et al.*, 1967). However, complexes formed between RNA polymerase and polynucleotides may have a different partial specific volume. Since the enzyme–polynucleotide solutions contained more than one molecular species of the enzyme as well as, in some cases, free polynucleotide, it was not possible to determine the partial specific volume of the complexes. In addition, the viscosity of buffer–polynucleotide solutions was not determined. Therefore, all sedimentation coefficients are given as observed (s_{20}).

Results

Our previous experiments with RNA polymerase, done under conditions of high ionic strength which promote dissociation, led to the suspicion that perhaps the 13S form of the enzyme had enzymatic activity (Ratliff *et al.*, 1967). However, we did not show that the enzyme was indeed dissociated in these experiments, and we could not rule out the possibility that DNA and/or some other component of the reaction mixture might cause a reassociation of the subunits. To investigate the size of the RNA polymerase molecule when

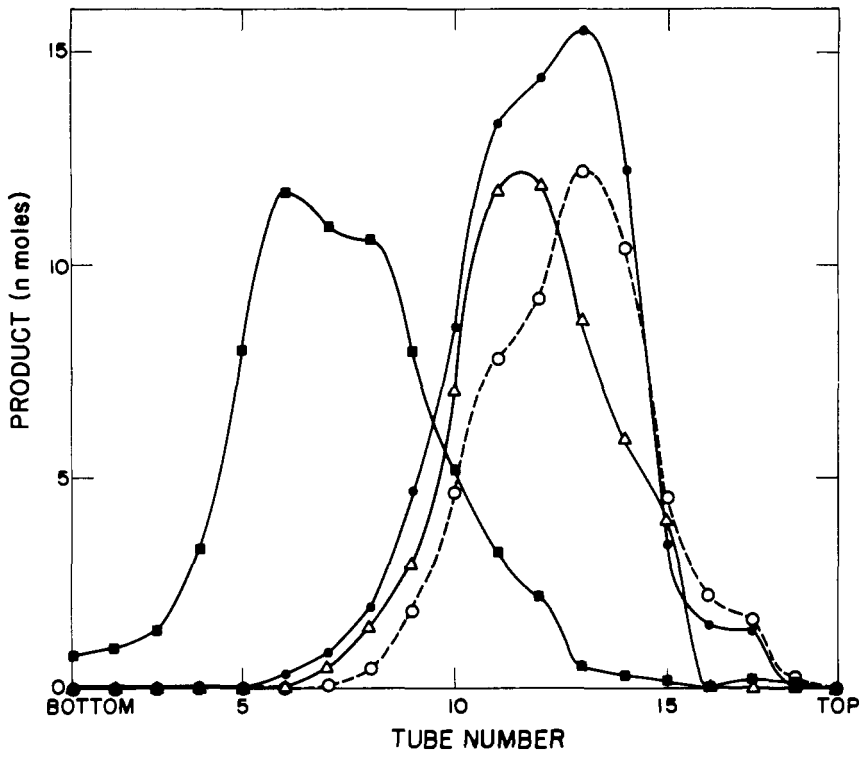

FIGURE 2: Sedimentation of RNA polymerase and RNA polymerase–polynucleotide mixtures in 5–15% glycerol gradients. RNA polymerase and polymerase–polynucleotide mixtures were layered over gradients and centrifuged as described under Materials and Methods. The fractions from each gradient were assayed using salmon sperm DNA as template. The labeled nucleoside triphosphate was ATP-8-^{14}C (3.6×10^5 cpm/μmole). The fractions from the gradient containing the polydeoxynucleotide were also assayed with UTP-2-^{14}C (3.8×10^5 cpm/μmole). (1) RNA polymerase (120 μg) alone, salmon sperm DNA assay (■—■); (2) RNA polymerase (120 μg) and tRNA (50 μg), salmon sperm DNA assay (△—△); (3) RNA polymerase and $d(pH)_3$-$(pA)_{50}$, salmon sperm DNA assay (○—○); and (4) assay with UTP-2-^{14}C (no additional template) (●—●).

complexed with a template, we mixed the enzyme with $d(pT)_{13}$[1] and centrifuged the mixtures through glycerol density gradients. Figure 1 shows the results of one of these experiments. In other experiments, it was found that the free enzyme sedimented at about 22 S in these gradients. The complex of RNA polymerase and $d(pT)_{13}$ sedimented somewhat slower than did the free enzyme. Furthermore, as the amount of $d(pT)_{13}$ added to the enzyme was increased, the complex sedimented even slower. In similar experiments, we found that other oligodeoxyribonucleotides such as $d(pC)_9$ also produced a slight decrease in sedimentation of the enzyme. On the other hand, large amounts of ATP did not affect sedimentation. Likewise, $d(pT)_3$, an oligodeoxynucleotide which does not serve as a template for the enzyme, did not affect its sedimentation rate.

Other experiments revealed that tRNA and longer polydeoxynucleotides, which presumably are more tightly bound to the enzyme, produced a large decrease in its rate of sedimentation. The decrease in sedimentation coefficient produced by these compounds was much greater than that produced by an equivalent amount of $d(pT)_{13}$. This is shown in Figure 2. Heparin, another high molecular weight anion, also produced a large decrease in sedimentation coefficient. Since heparin completely inhibits the enzyme, sedimentation of the polymerase–heparin complex was followed by determination of the protein in fractions from the density gradient centrifugation. In view of the very large change in sedimentation coefficient of RNA polymerase produced by tRNA and longer polydeoxynucleotides, we conclude that these compounds caused a dissociation of the enzyme.

In order to obtain more complete information on the template-induced dissociation of RNA polymerase and especially to study the dependence of this dissociation on template concentration, studies were done which utilized both analytical and density gradient centrifugation. In the first series of experiments, RNA polymerase was dialyzed against 0.1 M KCl–5.0 mM

[1] Abbreviations used: $d(pC)_n$, chemically synthesized polymer of deoxycytidylate, where n is the number of monomer units; $d(pT)_n$, chemically synthesized polymer of deoxythymidylate, where n is the number of monomer units; and $\bar{n}$, the estimated average number of monomer units in enzymatically lengthened polydeoxynucleotides; ATP, GTP, and UTP, adenosine, guanosine, and uridine triphosphates.

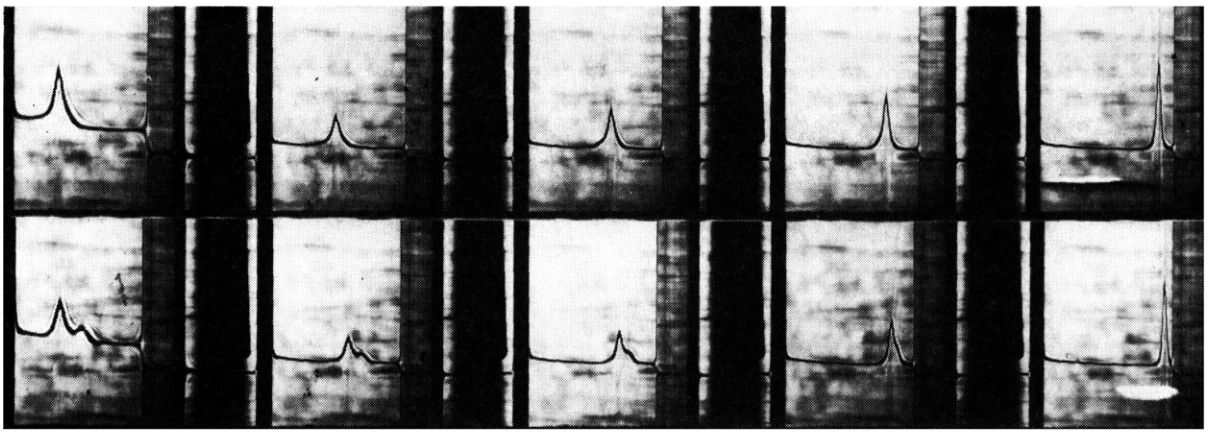

FIGURE 3: Analytical centrifugation of RNA polymerase and a RNA polymerase–polydeoxythymidylate mixture Top: RNA polymerase in 0.1 M potassium chloride–5.0 mM potassium phosphate (pH 7.5) (6 mg/ml) was centrifuged in a Spinco Model E ultracentrifuge as described in Materials and Methods. Sedimentation is from right to left. Pictures were taken 8 min apart using a phase-plate angle of 70° for the first four pictures and 50° for the fifth. Bottom: an excess of polydeoxythymidylate was added to the solution which was centrifuged in the top pictures, and the polymerase–polydeoxythymidylate solution was examined. The first four pictures were taken 8 min apart with a 70° phase-plate angle. The fifth picture was taken 16 min after the fourth using an angle of 50°.

potassium phosphate (pH 7.5). Figure 3 (top) shows the sedimentation pattern of the freshly dialyzed enzyme. The observed sedimentation coefficient was 23.2 S ($s_{20,w}$ = 23.7 S). After completion of this run, the contents of the ultracentrifuge cell were remixed by inversion, an aliquot was removed for density gradient centrifugation, and an excess of $d(pT)_{\bar{30}}$ was added. If the molecular weight of the enzyme (24 S) was 1×10^6 (Ratliff *et al.*, 1967), the molar ratio of $d(pT)_{30}$ to RNA polymerase in the ultracentrifuge cell was about 3. Because of uncertainties in the molecular weight of the enzyme (see Discussion) and the length of the polydeoxynucleotides, the mole ratios given could be as much as a factor of two from the true values. As soon as the rotor temperature again reached 20°, the sedimentation pattern of the polymerase–$d(pT)_{\bar{30}}$ complex was examined (Figure 3, bottom). Two peaks were observed with sedimentation coefficients of 18.9 and 13.5 S. Density gradient centrifugations were done on aliquots of the polymerase and polymerase–$d(pT)_{\bar{30}}$ solutions (Figure 4). Fractions from the polymerase–polydeoxythymidylate gradient centrifugation were assayed by incubation with ATP-8-^{14}C in the absence of any additional template. Two peaks of polyadenylate-forming activity were seen, both of which sedimented slower than did unbound RNA polymerase in the control tube. We conclude, therefore, that both the 18.9S and 13.5S peaks observed in the ultracentrifuge contained active RNA polymerase bound to polydeoxythymidylate. These results do not prove that the 13S and 19S forms as such are enzymatically active, since we cannot rule out the possibility of reassociation of the enzyme in the assay mixtures.

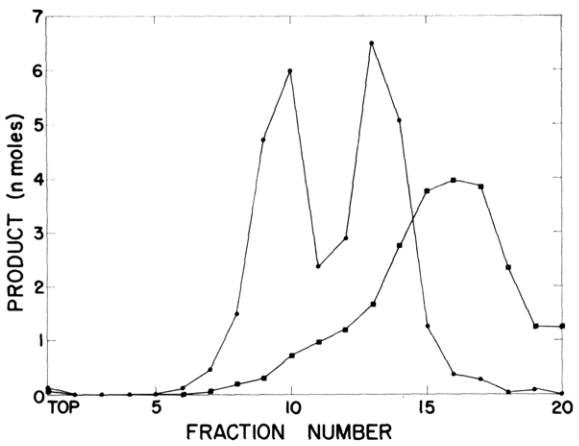

FIGURE 4: Density gradient centrifugation of solutions shown in Figure 3. Aliquots of the solutions used in the centrifugations shown in Figure 3 were sedimented through 5–15% glycerol gradients for 4.5 hr. Both gradient tubes contained 100 μg of protein. Fractions from the tube containing RNA polymerase only (■—■) were assayed with salmon sperm DNA as template. Incorporation of label was corrected so that the result is expressed in terms of total nucleotide polymerized. Fractions from the tube containing the RNA polymerase–$d(pT)_{30}$ (●—●) complexes were assayed by incubating with ATP-8-^{14}C without adding any other template.

In an extension of these experiments, different amounts of $d(pC)_{\bar{26}}$ were added to aliquots of the RNA polymerase solution. The protein–template complexes were then examined by ultracentrifugation. Figure 5 shows some of the results of the ultracentrifugal analysis. In a solution in which the mole ratio of template to enzyme was about 0.4, two main boundaries were seen (Figure 5, top). The faster boundary had an

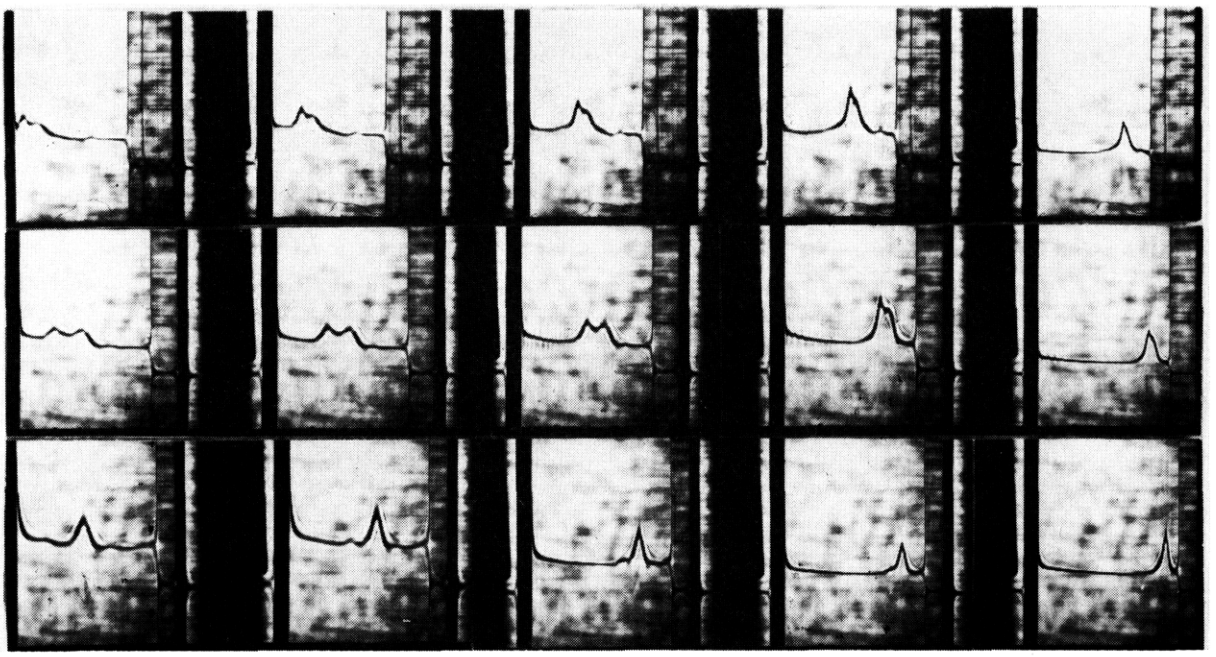

FIGURE 5: Analytical centrifugation of RNA polymerase–$d(pC)_{26}$ complexes. The solution of RNA polymerase used in the experiments shown in Figure 3 was diluted to 4.4 mg/ml. To aliquots of this solution were added different amounts of $d(pC)_{26}$. These mixtures were centrifuged as described in Materials and Methods. Top: the template to enzyme ratio was about 0.4. The pictures were taken 8, 16, 24, 32, and 40 min after reaching full speed. The phase-plate angle for the first picture was 70°; for subsequent pictures it was 50°. Middle: the template to enzyme ratio was about 3. The pictures were taken 8, 16, 32, 40, and 48 min after reaching full speed. The phase-plate angle for the first picture was 70°; for subsequent pictures it was 50°. Bottom: the template to enzyme ratio was about 18. The pictures were taken 8, 16, 24, 40, and 56 min after reaching full speed at respective phase-plate angles of 60, 60, 50, 40, and 40°.

observed sedimentation coefficient of 24.4 S, while that of the slower was 20.0 S. When the mole ratio was about 3, no 23S peak was observable (Figure 5, middle). Instead, two main peaks sedimenting at 19.3 and 13.2 S were seen. When a large excess of template was added to the enzyme (mole ratio 18), there was an almost complete conversion to the 13S form of the enzyme (Figure 5, bottom). Only a small peak sedimenting at 19 S was still observable. This boundary was still present at even higher template to enzyme ratios. Also in all three of the schlieren pictures shown in Figure 5, there was a small peak which sedimented at about 8.5 S. The latter is especially evident in the top schlieren picture in Figure 5. This run was done several days after the other experiments in which higher template to enzyme ratios were used. When the stock solution of RNA polymerase was reexamined at this time, it too was found now to contain a boundary which sedimented at 8.5 S, although none was present initially (Figure 3, top). We conclude that this small peak was formed as a consequence of the "aging" of the polymerase solution. Density gradient centrifugation revealed that it had no catalytic activity.

The data obtained from the schlieren pictures shown in Figure 5 are given in tabular form in Table I. It is clear that as the relative amount of template is increased the enzyme becomes more fully dissociated.

Density gradient centrifugations were done on all the polymerase–$d(pC)_{26}$ solutions. Fractions were assayed with GTP-8-^{14}C. An interesting observation from these experiments was that in no case were two peaks of polyguanylate-forming activity found. This activity invariably sedimented in the glycerol gradients at about 13–15 S. We believe that conditions in the glycerol

TABLE I: Sedimentation Data on RNA Polymerase and RNA Polymerase–$d(pC)_{26}$ Complexes.

Approximate Mole Ratio of $d(pC)_{26}$:RNA Polymerase	Observed Sedimentation Coefficients (S)[a]		
(Figure 3, top)	23.2		
0.4 (Figure 5, top)	23.4	20.0	
3 (Figure 5, middle)		19.2	13.1
18 (Figure 5, bottom)		19.3	13.2

[a] Neglecting the small boundary which sedimented at 8.5 S.

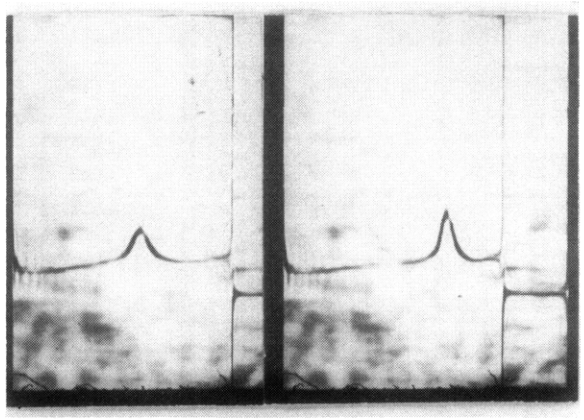

FIGURE 6: Analytical centrifugation of RNA polymerase in 80 mM Tris-HCl (pH 7.9), 4 mM $MgCl_2$, 1 mM $MnCl_2$, and 10 mM 2-mercaptoethanol. The protein concentration was 1.8 mg/ml. Sedimentation was from right to left. Pictures were taken 16 and 24 min after reaching full speed at a 60° phase-plate angle.

gradients enhance dissociation of the enzyme (D. A. Smith and A. M. Martinez, unpublished data). The data of Stevens *et al.* (1966) indicate that conditions in their sucrose density gradient solutions also stimulated dissociation of RNA polymerase. These observations, however, do not seem to be adequate to explain the existence of two peaks of polyadenylate-forming activity (Figure 4) in the polydeoxythymidylate–polymerase complexes when only one peak of polyguanylate-forming activity could be found in the $d(pC)_{\overline{26}}$–polymerase solutions. Furthermore, density gradient centrifugations of the RNA polymerase–$d(pT)_{\overline{22}}$ complexes described below revealed that, in some cases, two peaks of polyadenylate-forming activity could be detected while, in other cases, although two peaks were expected, only one (13–15 S) was observed.

In addition to the experiments in which RNA polymerase was dialyzed against 0.1 M KCl–5.0 mM potassium phosphate (pH 7.5), another series of experiments was done with a different preparation of RNA polymerase which had been dialyzed against 80 mM Tris-HCl (pH 7.9), 4.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, and 10 mM 2-mercaptoethanol. This is the basic solution used in assay of the enzyme. Aliquots of this solution were mixed with different amounts of $d(pT)_{\overline{22}}$ and analyzed in the ultracentrifuge. The results from these experiments were similar to those presented above (Figures 3–5) for polymerase–$d(pC)_{\overline{26}}$ complexes.

In the Tris buffer, freshly dialyzed RNA polymerase sedimented as a homogeneous peak with an observed sedimentation coefficient of 25.0 S (Figure 6). This is somewhat larger than the 23.2 S value found in potassium chloride–potassium phosphate. It may be due to the fact that the protein concentration in this experiment was only 1.8 mg/ml compared to 6.0 mg/ml previously. However, Fuchs *et al.* (1964) have reported that the dependence of sedimentation velocity on concentration of the enzyme is negligible. In the Tris buffer, RNA polymerase showed a tendency to aggregate. Three days after the initial determination of the sedimentation coefficient (Figure 6), the enzyme was again examined in the ultracentrifuge. At this time a second boundary comprising about 30% of the material was seen. The sedimentation coefficient of this peak was 31.2 S. Such aggregates have been observed previously (Fuchs *et al.*, 1964).

In order to see if the template-induced dissociation was reversible, DNase was added to a $d(pT)_{\overline{22}}$–polymerase solution in which the template to enzyme ratio was 5. Previously analysis in the ultracentrifuge revealed that this solution was composed of 19S (50%) and 13S (50%) peaks. After DNase digestion, the solution was again examined in the ultracentrifuge. The solution was now composed of 31S (50%) and 25S (50%) peaks. It seems, therefore, that the template-induced dissociation is entirely reversible. Furthermore, it seems obvious from our studies that polydeoxynucleotides cause dissociation of heavier aggregates as well as the 24S particle. Some of the results obtained in the second series of experiments are presented in Table II.

Discussion

Several investigations have dealt with the binding of DNA to *E. coli* RNA polymerase and the sedimentation properties of these complexes (Crawford *et al.*, 1965; Sternberger and Stevens, 1966; Richardson, 1966b). However, because several polymerase molecules bind to a single molecule of high molecular weight DNA, these studies could provide no information on the size of the polymerase molecule which is actually bound. In a recent study on the binding of tRNA to RNA polymerase, Bremer and Stent (1966) suggested, as a possible explanation of their findings, that reaction with RNA causes the enzyme to dissociate into subunits. Our approach to the problem of the size of the active polymerase molecule was to study the sedimentation properties of RNA polymerase when complexed with polydeoxynucleotides. The molecular weights of these oligomers were about 1% that of the 24S RNA polymerase molecule. Thus, they contributed very little to the size of the sedimenting complexes. Results obtained through the use of analytical and density gradient centrifugation showed that RNA polymerase when complexed to polydeoxynucleotides sedimented at a slower rate than did free 24S enzyme. As increasing amounts of template were added to the 24S form of the enzyme, boundaries which sedimented at about 19 and 13 S appeared. The relative amounts of each of these species were dependent upon the mole ratio of template to RNA polymerase. As this ratio was increased, the slower sedimenting species became predominant.

The 13S form of the enzyme is obviously a dissociation product of a higher aggregate form, and the same is most likely true for the 19S species. There is a slight possibility, however, that 23S RNA polymerase when complexed with a short template could greatly alter

TABLE II: Sedimentation Data on RNA Polymerase and RNA Polymerase–d(pT)$_{22}$ Complexes.

Approx Mole Ratio of d(pT)$_{22}$:RNA Polymerase	Obsd Sedimentation Coefficients (S)[a]			
		25.0		
b	31.2 (30)	25.2 (70)		
5			18.8 (50)	13.1 (50)
5 + DNase[c]	31.5 (50)	24.6 (50)		

[a] The numbers in parentheses in these columns present approximate percentages of the total area under the schlieren peaks. [b] This ultracentrifugal analysis was done 3 days later than that shown on the first line. [c] The polymerase–template solution was made 0.8 mM in $CaCl_2$, and 10 μg of DNase I and 10 μg of micrococcal DNase were added. After incubation at 20° for 6 hr, the solution was again examined in the ultracentrifuge.

its shape and so produce a particle with a sedimentation coefficient of 19 S. Further studies will be necessary to resolve this point. On the basis of their electron microscopic studies of RNA polymerase, Colvill *et al.* (1966) suggested that the 25S species of the enzyme which they observed in density gradients might be formed by dimerization of two 18S molecules. Zillig *et al.* (1966) suggested that the 18S species is a dimer of a 12S form and that the 24S species is a trimer. They believe the molecular weight of the 24S species to be about 600,000. Richardson (1966a) observed species which sedimented at about 21 and 13 S. Sedimentation equilibrium studies of each of these two forms of the enzyme yielded estimated molecular weights of 440,000 ± 80,000 for the 13S form and 880,000 ± 160,000 for the 21S form. The molecular weight of 24S RNA polymerase as determined by the approach to equilibrium technique was about 1×10^6 (Ratliff *et al.*, 1967). Until the diverse and conflicting reports regarding the molecular weight and degree of aggregation of the enzyme can be resolved, one can only speculate about the stoichiometry of the template-induced dissociation observed in this work.

Although polynucleotides can cause dissociation of RNA polymerase, the question of the size of the active enzyme was not resolved since we cannot rule out the possibility of reassociation of the subunits in the assay mixtures. However, we believe that available evidence strongly suggests that the 13S form of the enzyme is active, although it probably is not the only active form. Zillig *et al.* (1966) found that RNA polymerase could be reactivated by exposing it to high ionic strength solutions (dissociating conditions). We found that with salmon sperm DNA as template the activity of the enzyme is increased under conditions of ionic strength which cause its dissociation (Ratliff *et al.*, 1967). M. J. Chamberlin (personal communication) recently described experiments in which the initiation of RNA chains was followed by incorporation of γ-^{32}P-labeled nucleoside triphosphate. Under conditions of these experiments, it appeared that each polymerase molecule could start only one RNA chain. On this basis, he calculated that the active molecule had a molecular weight in the range of 400,000–500,000. All of these experiments, together with the findings in this report which show that template can cause dissociation of RNA polymerase, support the hypothesis that of the many molecular forms of RNA polymerase the 13S form has the highest specific activity.

References

Bollum, F. J., Groeniger, E., and Yoneda, M. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 853.

Bremer, H., and Stent, G. (1966), *Acta Biochim. Polon. 13*, 367.

Colvill, A. J. E., Van Bruggen, E. F. J., and Fernández-Morán, H. (1966), *J. Mol. Biol. 17*, 302.

Crawford, L. V., Crawford, E. M., Richardson, J. P., and Slayter, H. S. (1965), *J. Mol. Biol. 14*, 593.

Fuchs, E., Zillig, W., Hofschneider, P. H., and Preuss, A. (1964), *J. Mol. Biol. 10*, 546.

Hayes, F. N., Mitchell, V. E., Ratliff, R. L., Schwartz, A. W., and Williams, D. L. (1966), *Biochemistry 5*, 3625.

Hayes, F. N., Mitchell, V. E., Ratliff, R. L., and Williams, D. L. (1967), *Biochemistry* (in press).

Kato, K., Gongalves, J. M., Houts, G. E., and Bollum, F. J. (1967), *J. Biol. Chem. 242*, 2780.

Khorana, H. G., Turner, A. F., and Vizsolyi, J. P. (1961), *J. Am. Chem. Soc. 83*, 686.

Khorana, H. G., and Vizsolyi, J. P. (1961), *J. Am. Chem. Soc. 83*, 675.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Ratliff, R. L., Smith, D. A., and Trujillo, T. T. (1967), Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico, Report LA-3638.

Richardson, J. P. (1966a), *Proc. Natl. Acad. Sci. U. S. 55*, 1616.

Richardson, J. P. (1966b), *J. Mol. Biol. 21*, 83.

Smith, D. A., Ratliff, R. L., Trujillo, T. T., Williams, D. L., and Hayes, F. N. (1966), *J. Biol. Chem. 241*, 1915.

Smith, D. A., Ratliff, R. L., Williams, D. L., and

Martinez, A. M. (1967), *J. Biol. Chem. 242*, 590.
Sternberger, N., and Stevens, A. (1966), *Biochem. Biophys. Res. Commun. 24*, 937.
Stevens, A., Emery, A. J., Jr., and Sternberger, N. (1966), *Biochem. Biophys. Res. Commun. 24*, 929.
Zillig, W., Fuchs, E., and Millette, R. (1966), *in* Procedures in Nucleic Acid Research, Cantoni, G. L., and Davies, D. R., Ed., New York, N. Y., Harper & Row, p 323.
Zubay, G. (1962), *J. Mol. Biol. 4*, 347.

Glycoproteins of Ehrlich Ascites Carcinoma Cells. Incorporation of [^{14}C]Glucosamine and [^{14}C]Sialic Acid into Membrane Proteins*

Janos Molnar

ABSTRACT: Washed Ehrlich ascites carcinoma cells were incubated with labeled glucosamine which resulted in the appearance of radioactive glucosamine and sialic acid in trichloroacetic acid insoluble cell constituents. The subcellular localization of the products of this reaction have been investigated by separating the labeled cell homogenates to nuclei, mitochondrial, rough and smooth microsomal, and supernatant fractions by using differential and density centrifugation methods. The fractions were characterized in terms of protein, hexosamine, sialic acid, radioactivity, ribonucleic acid, deoxyribonucleic acid, and also by electron microscopy. The smooth and rough microsomal fractions were analyzed further by sucrose density gradient centrifugation and a few enzymatic activities were also determined. Three methods were tested for separating subcellular particles. By the best method more than 40% of the cellular hexosamine, sialic acid, and radioactivity could be isolated with the smooth microsomal fraction.

The data supported the view that most of the sialic acid and hexosamine of Ehrlich cells is localized in the plasma membrane and smooth endoplasmic reticular membrane structures. Incorporation of glucosamine occurred mainly into the macromolecules containing these constituents.

In a previous paper it was shown that glucosamine-^{14}C was readily incorporated into proteins by Ehrlich ascites carcinoma cells (Molnar *et al.*, 1965a). Two major types of products were observed. One appeared in the incubation media (*in vitro*), or in the ascites plasma (*in vivo*), as a heterogeneous mixture of glycoproteins (Molnar *et al.*, 1965b) and the other remained associated with cellular elements. The "secreted macromolecules" contained about equal amounts of radioactivity in their galactosamine and glucosamine moieties and in a hitherto unidentified compound. The cellular elements, on the other hand, had radioactivity mainly in glucosamine and about 10% of the total activity was in sialic acid.

The present paper describes our attempts to find the subcellular localization of the cellular products of glucosamine-^{14}C incorporation. We were guided in these experiments by the observations of Wallach and his co-workers (1961, 1966) who reported that 73% of sialic acid of Ehrlich cells could be released by treatment of intact cells with neuraminidase with a concomitant decrease of electrophoretic mobility. These experiments and some others suggested that about 70% of the cellular sialic acid is located on the outer surface of plasma membranes. A similar conclusion was reached by Langley and Ambrose (1964, 1967) who subjected Ehrlich cells to limited digestion by trypsin and found that about 70% of sialic acid was released with mucopeptides which also contained galactosamine. These experiments suggested that the plasma membrane fraction, if separable from the other membranes, should contain about 70% of cellular sialic acid and hexosamine. Since both of these sugar moieties became labeled in our experiments we were expecting that about 70% of the incorporated radioactivity should be present in the plasma membrane fraction too, unless some of the products localized in other types of structural elements have higher, or lower, turnover rates.

It will be shown that by our best method 40–50%

* From the Department of Biological Chemistry, College of Medicine, University of Illinois, Chicago, Illinois. *Received June 5, 1967.* This investigation was supported by research grants from the American Cancer Society, ACS P-406 and ACS 1N9G #4, and also by U. S. Public Health Service Grants PHS 5 RO1 MHD 02591-02 and GRSG 153. The author is a U. S. Public Health Service Career Development Awardee (No. 5-K3-CA-11, 158-03).