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Studies of Nucleotide Binding to the Ribonucleic Acid Polymerase by Equilibrium Dialysis*

C. W. Wu and D. A. Goldthwait†

ABSTRACT: The interaction of nucleoside triphosphates with ribonucleic acid polymerase of *Escherichia coli* has been studied by the technique of equilibrium dialysis. In the absence of divalent metal, purine nucleotides bind to a single site on the enzyme (mol wt 370,000). Dissociation constants for guanosine triphosphate of 0.15 mM and for adenosine triphosphate of 0.17 mM were observed. Rifamycin inhibited completely the binding of purine nucleoside triphosphate in the absence of divalent metal. No binding of pyrimidine nucleoside triphosphates at concentrations up to 0.2 mM was observed in the absence of divalent metal. With 10 mM $MgCl_2$, two binding sites for purine nucleoside triphosphates were observed. The weak site with a K_s of 0.15 mM was abolished by rifamycin leaving a single strong binding site with a K_s of 0.015 mM. A single weak binding site for pyrimidine nucleoside triphosphates was observed with a K_s for cytidine triphosphate of 0.23 mM and for uridine triphosphate of 0.37 mM. This binding was not sensitive to rifamycin.

There was no enhancement of pyrimidine nucleoside triphosphate binding by guanosine triphosphate, or Mn, or at a lower pH. Some decrease in the K_s for uridine triphosphate was observed with transfer ribonucleic acid, and for cytidine triphosphate with polyadenylic acid. Uridine monophosphate was not bound. No evidence of phosphodiester bond formation was observed.

The interaction between nucleoside triphosphates and the DNA-dependent RNA polymerase has been studied in our laboratory with several approaches. In kinetic experiments (Anthony *et al.*, 1969a), it has been concluded that a low apparent K_m exists for the process of polymerization and a high apparent K_m for the process of initiation. The apparent K_m for polymerization is approximately 0.015 mM. The apparent K_m for initiation is approximately 0.15 mM or one order of magnitude higher than that for polymerization. This difference in K_m values suggests that there might be at least two binding sites with different affinities for nucleoside triphosphates on the RNA polymerase, one for initiation and the other for polymerization.

Evidence that there is an initiation site on the enzyme responsible for the binding of the 5′-terminal nucleoside triphosphates has been obtained in the absence of DNA by a fluorescence-quenching technique (Wu and Goldthwait, 1969) which is based on the ability of nucleotides to decrease the fluorescence of tryptophan residues in the RNA polymerase. This site binds purine nucleotides preferentially to

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pyrimidine nucleotides, which is in accord with the fact that the 5′-terminal nucleotide residues of RNA synthesized *in vitro* by the DNA-dependent RNA polymerase are primarily purine nucleotides (Maitra and Hurwitz, 1965; Maitra *et al.*, 1967). The binding constant for purine nucleoside triphosphates observed by this technique is similar to the apparent K_m of initiation. Moreover, the antibiotic rifamycin which is a specific inhibitor of initiation of RNA synthesis (Sippel and Hartmann, 1968), also interacts with this site on the enzyme.

No polymerization site has been observed in fluorescence studies. This could be because no such binding site exists in the absence of DNA, or because binding does occur but there is no tryptophan residue near the site which can be observed by the fluorescence-quenching technique.

The present investigation with equilibrium dialysis was undertaken to confirm the presence of the initiation site demonstrated by the fluorescence technique and to search for the polymerization site on the enzyme suggested by the kinetic studies.

Materials and Methods

Biochemicals. Unlabeled nucleoside triphosphates were purchased from Sigma Chemical Co. and tritiated nucleoside triphosphates from Schwarz BioResearch, Inc. [γ-^{32}P]GTP, -CTP, and [α-^{32}P]UMP were purchased from International Chemical and Nuclear Corp. *E. coli* tRNA (strain B) was obtained from General Biochemicals and polyadenylic acid from the Miles Chemical Co.

Micrococcus lysodeikticus DNA was obtained from the Miles Chemical Co. Calf thymus DNA was isolated by Dr. George Becking. Rifamycin SV was a gift from Dr. J. W. Corcoran.

RNA Polymerase. E. coli RNA polymerase was prepared from *E. coli* MRE 600 or *E. coli* W according to the procedure of Maitra and Hurwitz (1967) with omission of the calcium phosphate gel absorption step. The enzyme was purified further by Agarose (Bio-Gel A 1.5 M) gel filtration chromatography. The enzyme thus prepared incorporated 6000–7200 mμmoles of one labeled NTP per milligram of protein per hour with calf thymus DNA. It showed a single peak by sedimentation-velocity ultracentrifugation and a molecular weight of 370,000 was obtained in 0.5 M KCl buffer from a sedimentation equilibrium study by the method of Yphantis (1964) (Figure 1). The enzyme was stored in 30% saturated ammonium sulfate solution at 4°.

RNA Polymerase Assay–Pyrophosphate Release. RNA polymerase activity was estimated by the incorporation of [^{3}H]CTP (1400 cpm/mμmole) into trichloroacetic acid insoluble material as described (Anthony *et al.*, 1969a). PP_i release from [γ-^{32}P]GTP and -UTP was determined by the method reported by Maitra and Hurwitz (1967), with some modification.

Desalting of the Enzyme Solution. A continuous-flow dialysis cell was designed for the purpose of desalting a small volume of the enzyme solution. Its structure was similar to an equilibrium dialysis cell with two chambers (150 μl each in volume) separated by a semipermeable membrane. One chamber contained the enzyme solution. A buffer passed from a reservoir through small Tygon tubing to the second chamber and was finally collected in a beaker.

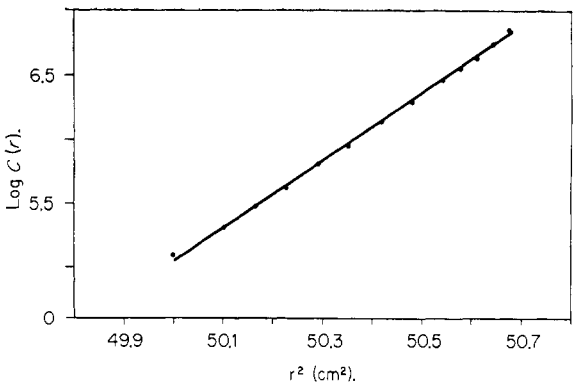

FIGURE 1: Sedimentation equilibrium study of RNA polymerase. RNA polymerase, stored in 30% $(NH_4)_2SO_4$, was passed through a Bio-Gel A-15M column (2.5 × 55 cm) previously equilibrated with 0.5 M KCl, 0.05 M Tris-HCl (pH 7.9), and 0.001 M mercaptoethanol. The protein concentration was adjusted to 0.4 mg/ml for analysis. A double-sector interference cell (Epon 12 mm) was used with sapphire windows. The sample channel of the cell contained 0.1 ml of the enzyme solution and the 0.01 ml of fluorocarbon FC-43 (Minnesota Mining and Manufacturing Co.). The reference channel contained 0.12 ml of the appropriate buffer. The height of the sample column was 3 mm. The run was at 5.97° for 22 hr with a rotor speed of 11,000 rpm. The Rayleigh interferometric system was used and the attainment of sedimentation equilibrium was judged by identical values of the vertical deflection of the fringes measured on a series of photographs taken after 20 hr at intervals of 1 hr.

Before the binding experiments 0.15 ml of the enzyme solution (stored in 30% ammonium sulfate) was dialyzed against a continuous flow of 0.15 M KCl buffer (0.15 M KCl–0.05 M Tris-HCl, pH 7.9, 10^{-3} M mercaptoethanol, and 10^{-4} M EDTA) for 3 hr. The cell was rotated slowly on a Super-Mixer with a velocity control during the dialysis. The ammonium sulfate in the enzyme solution was removed after 3 hr as estimated by conductivity measurement.

Equilibrium Dialysis. The cells used in equilibrium dialysis experiments were originally designed by Dr. Arthur Kornberg and Dr. Paul Englund. They were made from Plexiglass and the volume of each chamber was about 25 μl. The membranes were standard Visking dialysis tubing (size 20), which was stretched by the method of Craig and King (1962). After stretching, the membranes were washed several times with 0.01 N acetic acid, rinsed thoroughly with distilled water, and then stored in 0.15 M KCl buffer at 4° for use.

The equilibrium dialysis cells were loaded with a 25-μl Hamilton syringe (20 μl/chamber) and aliquots were removed with a 10-μl syringe (5 μl each time). Chimney adaptors attached to the syringe were used to improve the accuracy and the repeatability of the pipetting.

After loading 20 μl of protein on one side and 20 μl of labeled triphosphates on the other, the cells were sealed with Scotch tape and tightened with a screw in a metal holder. They were then shaken gently on a shaker at 25°. Stirring was by a 1-mm diameter glass bead which had been placed in each chamber.

After equilibrium was reached (90 min), two 5-μl aliquots were pipetted from each chamber into scintillation vials and 0.6 ml of 1 N NH_4OH and 10 ml of liquid scintillant were added to each vial. The vials were then counted in a Packard TriCarb scintillation counter.

Estimation of Equilibration Time. In a preliminary experi-

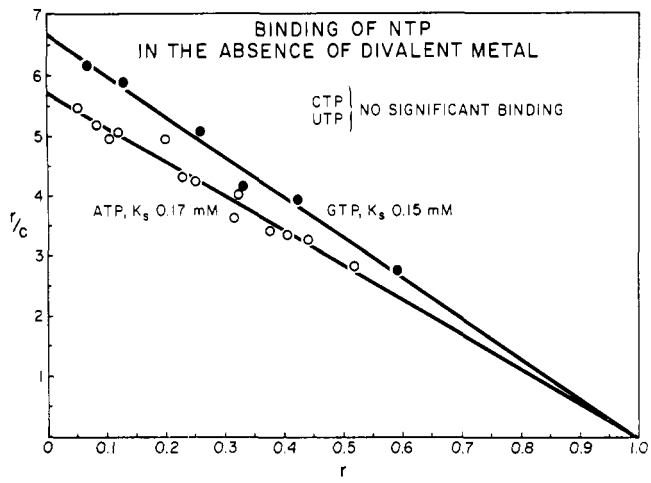

FIGURE 2: A Scatchard plot of nucleoside triphosphate binding by RNA polymerase in the absence of divalent metal. Data from two separate experiments are combined. The solvent contained 0.15 M KCl, 0.05 M Tris-HCl (pH 7.9), and 1 mM mercaptoethanol. No divalent metal was added and 0.2 mM EDTA was present to remove trace metal. Procedures and data analysis are described in Materials and Methods. Lines were drawn through $r = 1.0$.

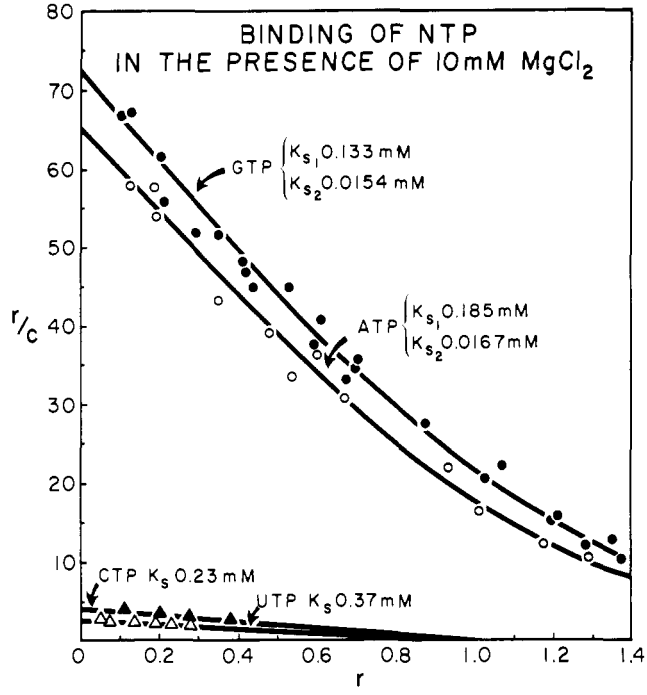

FIGURE 3: A Scatchard plot of nucleoside triphosphate binding by RNA polymerase in the presence of 10 mM $MgCl_2$. Data from three separate experiments are combined. The solvent contained 0.15 M KCl, 0.05 M Tris-HCl (pH 7.9), 1 mM mercaptoethanol, and 10 mM $MgCl_2$. Two theoretical curves are included for the cases of one strong and one weak site. These were determined from eq 1 and 4 the cited values for the intrinsic binding constants.

ment, the equilibration of labeled nucleotide in the absence of enzyme was examined under otherwise similar conditions. After 60 min, the radioactivities of both chambers were practically the same (within 3%). This result excluded any intrinsic barrier to the passage of nucleotides through the membrane.

Determination of NTP[1] *and Protein Concentrations.* NTP concentration in each chamber was calculated from the radioactivity counted and the specific activity of the NTP. A specific absorbance of 0.67 mg/ml at 280 mμ was used to determine the enzyme concentration before the dialysis. After dialysis, the $OD_{280\ 280\ m\mu}$ due to protein was estimated by ($OD_{280\ 280\ m\mu}$ measured) − (OD_{280} NTP). The OD_{280} NTP was calculated from the concentration of NTP measured by radioactivity and the molar absorbance of NTP at 280 mμ (also obtained experimentally). A molecular weight of 370,000 daltons for RNA polymerase was used in all calculations and was determined as noted above. The concentrations of RNA polymerase used in the binding experiments varied from 7 to 26 mg per ml (0.019–0.070 mM) and the NTP concentrations from 0.0025 to 0.15 mM.

Analysis of Equilibrium Dialysis Data. The equilibrium dialysis data were analyzed according to the general form of Scatchard's equation (Scatchard, 1949; Velick *et al.*, 1960)

$$\frac{r_i}{c} = n_i K_i - r_i K_i \qquad (1)$$

where c is the concentration of free NTP. n_i is the number of sites of type i corresponding to a microscopic binding constant of K_i, and r_i is the average number of moles of NTP bound by sites of this type per mole of enzyme.

If n designates the total number of NTP binding sites and r is the average number of moles of NTP bound per mole of enzyme, then

$$n = \Sigma n_i \qquad (2)$$

$$r = \Sigma r_i \qquad (3)$$

and

$$\frac{r}{c} = \Sigma \frac{r_i}{c} = \Sigma \frac{K_i n_i}{1 + K_i c} \qquad (4)$$

When all the sites are homologous, eq 1 may be simplified in terms of a single intrinsic binding constant, K

$$\frac{r}{c} = nK - rK \qquad (5)$$

Plotting of r/c *vs.* r will yield a straight line with the intercept on the ordinate equal to nK and the intercept on the abscissa equal to n. If more than one class of binding sites are present, then the plot will show pronounced curvature and the data may be fitted by an appropriate choice of constants with eq 1 and 4.

Results

Binding of Nucleotides by RNA Polymerase in the Absence of Divalent Metal. Figure 2 shows the Scatchard plot of the

[1] Abbreviation used is: NTP, nucleoside triphosphate.

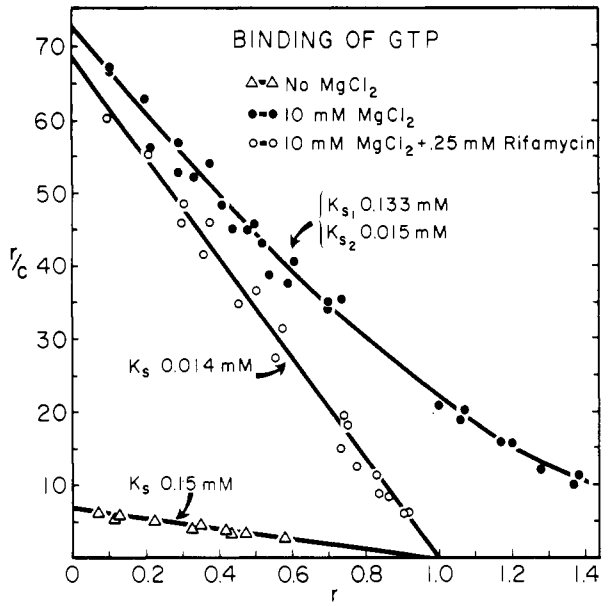

FIGURE 4: A Scatchard plot showing the effect of rifamycin SV on GTP binding by RNA polymerase with 10 mM $MgCl_2$. Data from two separate experiments are combined. The solvent contained 0.15 M KCl, 0.05 M Tris-HCl (pH 7.9), 1 mM mercaptoethanol, 10 mM $MgCl_2$, and 0.25 mM rifamycin SV. Some data from experiments with $MgCl_2$ and without rifamycin were taken from Figure 3 and some data without $MgCl_2$ from Figure 2.

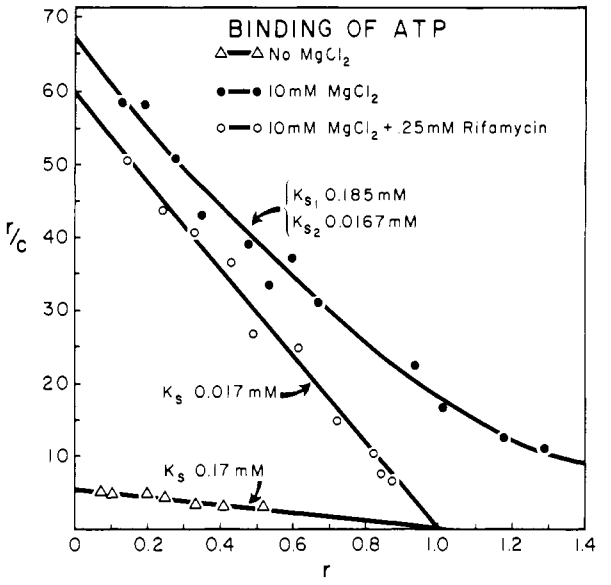

FIGURE 5: A Scatchard plot showing the effect of rifamycin SV on ATP binding by RNA polymerase. The solvent was the same as for Figure 4. Some data with $MgCl_2$ and without rifamycin are taken from Figure 3 and some data without $MgCl_2$ from Figure 2.

binding of NTP by RNA polymerase in the absence of divalent metal and the presence of 0.2 mM EDTA. Linear plots were obtained for both GTP and ATP with an n value equal to 1. The dissociation constants calculated from the plots were 0.15 mM for GTP and 0.17 mM for ATP. Since it was experimentally difficult to obtain points with r values higher than 0.6, very weak bindings (*e.g.*, dissociation constant one order of magnitude higher than 0.15 mM) cannot be ruled out. Similar concentrations of CTP and UTP showed no significant binding under these experimental conditions.

Binding of Nucleotides by RNA Polymerase in the Presence of 10 mM $MgCl_2$. Magnesium ions are required for the enzymatic activity of RNA polymerase. The optimal concentration of Mg^{2+} is 10 mM (Furth *et al.*, 1962) and with this concentration more than 99% of the NTP is in the form of Mg-NTP^{2-} complex (Sillen and Martell, 1964). The Scatchard plots of NTP binding by RNA polymerase in the presence of 10 mM $MgCl_2$ are demonstrated in Figure 3. CTP and UTP showed linear plots with $n = 1$. The dissociation constants were 0.23 mM for CTP and 0.37 mM for UTP.

The plot of purine nucleoside triphosphate binding had a pronounced curvature convex to the abscissa. It is impossible to fit curves of this form by assuming a single class of binding sites. The curves could, however, be fitted on the assumption of a strong binding site with a dissociation constant of 0.015 mM for GTP or 0.017 mM for ATP, and a weak binding site with a dissociation constant of 0.13 mM for GTP or 0.18 mM for ATP.

Effect of Rifamycin on Nucleotide–RNA Polymerase Binding. When an excess of rifamycin (0.25 mM, about ten times the concentration of enzyme) was present in the binding system without divalent metal, it abolished the binding of purine nucleotides by RNA polymerase. Similar inhibition was found of the binding of GTP or ATP to the weak binding site in the presence of 10 mM $MgCl_2$. This is demonstrated in Figure 4 for GTP and in Figure 5 for ATP. With addition of 0.25 mM rifamycin SV, the Scatchard plot showed only a single binding site with a dissociation constant of 0.015 mM. The weak binding site with a K_s of 0.15 mM disappeared. In other words, rifamycin only inhibits the weak binding and has no effect on the strong binding site. The pyrimidine binding by the enzyme in the presence of $MgCl_2$ was insensitive to rifamycin.

Binding of Pyrimidine Nucleotides under Various Conditions. In the presence of $MgCl_2$ and rifamycin, the purine NTP's bind to the enzyme with a dissociation constant comparable with the apparent K_m of polymerization. No similar binding has been observed for pyrimidine nucleotides. A number of attempts were made to increase the pyrimidine binding ability of the enzyme.

Several experiments were done, based on the assumption that binding of nucleotide at the first site might induce the formation of the second site. When 0.2 mM of GTP was present, the binding of UTP by RNA polymerase was decreased, while the same concentration of dATP, dAMP, or GMP had no significant effect on UTP–RNA polymerase binding. Thus, purine nucleotides, tested under these conditions did not induce a pyrimidine binding site which could be observed.

Formation of the second site secondary to binding of the template was also considered. *E. coli* tRNA was found to enhance the binding of UTP by the enzyme. The dissociation constant for UTP was lowered from 0.37 mM without tRNA to 0.084 mM with tRNA (2.5 mg/ml). Similarly, when 5 mg/ml of polyadenylate was added instead of tRNA and the binding of CTP by RNA polymerase was examined, the dissociation constant was lowered from 0.34 to 0.08 mM. The significance of this enhancement in binding of pyrimidine NTP will be discussed.

TABLE I: $^{32}PP_i$ Release from[γ-^{32}P]NTP's during Equilibrium Dialysis Studies.[a]

NTP	Expt	Condition	mμmoles of $^{32}PP_i$ Release
[γ-^{32}P]GTP	1	No enzyme	0.05
	2	No $MgCl_2$	0.06
	3	10 mM $MgCl_2$	0.06
	4	10 mM $MgCl_2$ + 0.25 mM rifamycin	0.05
	5	10 mM $MgCl_2$ + all four NTP's (0.1 mM) + ML-DNA (5 μg)	2.10
[γ-^{32}P]CTP	6	No enzyme; no poly A	0.03
	7	No enzyme + 5 mg/ml of poly A	0.03
	8	No poly A	0.04
	9	5 mg/ml of poly A	0.04
	10	5 mg/ml of poly A + 0.25 mM rifamycin	0.04
	11	5 mg/ml of poly A + all four NTP's (1.1 mM)	2.00

[a] The procedures of equilibrium dialysis were as described in Materials and Methods. The concentration of [γ-^{32}P]GTP and [γ-^{32}P]CTP used in each experiment was 0.1 mM. In expt 6–11, 10 mM $MgCl_2$ was present. The enzyme concentration was 0.038 mM (14.1 mg/ml) in expt 2–4 and 8–10 and 0.019 mM in expt 5 and 11. After the attainment of equilibrium, 10-μl aliquots were removed from each chamber, treated with 0.4 ml of cold 5% trichloroacetic acid, and 5 μl of 0.1 M sodium pyrophosphate and 0.15 ml of 1% bovine serum albumin were added. The mixtures were centrifuged and the supernatants were treated with 0.15 ml of 10% Darco G-60 solution at 0° for 5 min. The charcoal treatments were repeated twice and after centrifugation the supernatants were extracted several times with ether to remove the trichloroacetic acid. The samples were then dried, taken up with 0.5 ml of H_2O, counted in a scintillation system, and the amount of $^{32}PP_i$ was calculated for the 40-μl volume of the dialysis system.

Replacement of 10 mM $MgCl_2$ by 2 mM $MnCl_2$, or a shift of the pH to 7.0 from 7.9 did not increase the pyrimidine NTP binding by RNA polymerase. Also, unlike DNA polymerase which has been shown to bind pyrimidine mononucleotides (Kornberg, 1969), no significant binding of UMP to RNA polymerase was detected.

Evidence That No Phosphodiester Bond Formed during Equilibrium Dialysis. In order to prove that no polyribonucleotide or oligoribonucleotide was synthesized during the equilibrium dialysis studies, [γ-^{32}P]GTP plus enzyme and [γ-^{32}P]CTP plus enzyme plus poly A were used. If any phosphodiester bond was formed, $^{32}PP_i$ would be released from [γ-^{32}P]NTP and could be detected by its radioactivity. The results of these experiments are shown in Table I. With GTP no significant amount of $^{32}PP_i$ was detected either in the presence or absence of $MgCl_2$. The amount of PP_i released in the above two conditions was about the same as that observed in control experiments with no enzyme or with enzyme plus an excess of rifamycin. An experiment with all four NTP's and a small amount of *M. lysodeikticus* DNA was done as a control. Similar results were obtained with CTP and poly A.

In accord with the pyrophosphate release experiments, when the enzyme–NTP complex after equilibrium was dissociated with 5% trichloroacetic acid, and the bound NTP was subjected to thin-layer chromatography on polyethylimine cellulose plates in the solvent system of LiCl (Randerath and Randerath, 1967), a single peak identical with that of the free NTP was observed. No radioactivity was detected at the origin.

Discussion

In the present investigation, we have observed two different NTP binding sites on DNA-dependent RNA polymerase. This is summarized in Table II. The first site was demonstrated both in the presence and absence of divalent metal. This site binds purine but not pyrimidine nucleotides, binds them in the absence of divalent metal, possesses a dissociation constant equivalent to the apparent K_m of initiation obtained by kinetic studies, and is inhibited by rifamycin, a specific inhibitor of initiation. There is one binding site per molecule of mol wt. 370,000 A binding site or sites with similar properties were demonstrated with the fluorescence-quenching technique (Wu and Goldthwait, 1969). We propose that this binding site is for the terminal 5′-nucleoside triphosphate and represents the initiation site.

The nature of the second site is more complicated. It requires dilvaent metal and is not inhibited by rifamycin. Although the dissociation constant is comparable with the apparent K_m of polymerization, this dissociation constant is observed only with purine nucleotides. It is to be recalled that the apparent K_m of polymerization is 0.015 mM for both purine and pyrimidine nucleoside triphosphates (Anthony *et al.*, 1969a). In the presence of 10 mM $MgCl_2$ the pyrimidine nucleotides bind very weakly (K_s = 0.3–0.5 mM) to the RNA polymerase and this binding is insensitive to rifamycin. Whether the pyrimidines bind to the same site to which the purine nucleotides bind is not known.

If the second site to which purine NTP's binds in the presence of $MgCl_2$ is a polymerization site, then this raises the question why pyrimidine NTP's cannot bind to this site? One possible explanation is that NTP binds to the polymerization site only when the initiation site has already been occupied. In other words, the reason purine NTP's are able to bind to the polymerization site is because this site is induced by the nucleotide (or rifamycin) bound to the initiation site. Since pyrimidine NTP's cannot bind to the initiation site, their binding to the polymerization site is very weak or negligible. To test this possibility, the binding of UTP by RNA polymerase was examined in the presence of GTP. The binding was less than that without GTP. This could be due to the competition of GTP with UTP on the same site. However, GMP which also binds at the initiation site (Wu and Goldthwait, 1969) did not affect the binding of UTP. dATP and dAMP also had no effect on UTP–enzyme binding. Finally, no binding of UMP to the enzyme was observed.

TABLE II: Summary of Equilibrium Dialysis Studies.

$MgCl_2$ (mM)	Purine NTP	First Site K_s (mM)	First Site Rifamycin	Second Site K_s (mM)	Second Site Rifamycin
0	GTP	0.15	Inhibition	No binding	
	ATP	0.17	Inhibition	No binding	
10	GTP	0.14	Inhibition	0.015	No effect
	ATP	0.17	Inhibition	0.016	No effect

$MgCl_2$ (mM)	Pyrimidine NTP	K_s (mM)	Rifamycin
0	CTP	No binding	
	UTP	No binding	
10	CTP	0.37	No effect
	UTP	0.50	No effect

Another possible reason for the absence of pyrimidine NTP binding to a polymerization site is that it requires the template site to be occupied. DNA cannot be used for this purpose since it will act as a template for the DNA-dependent synthesis of homopolymers in the presence of a single NTP. tRNA is known to compete with DNA in its binding to RNA polymerase (Anthony *et al.*, 1969b). It is possible that the increased binding of UTP by the enzyme in the presence of tRNA is due to the occupation of the template site by tRNA. However, the possibility that tRNA might also serve as a template for polymerization cannot be ruled out. Natural and synthetic RNA templates have been reported to direct RNA synthesis with RNA polymerase (Fox *et al.*, 1964). In order to avoid the template function, polyadenylate was used instead of tRNA and the binding of CTP (a noncomplementary nucleotide) by the enzyme was examined. As shown in the results, polyadenylate also significantly increased the CTP binding. This might imply that the pyrimidine NTP binding to the polymerization site requires the presence of either a template or a template analog which binds to the template site on the enzyme.

It has been reported by several investigators (Smith *et al.*, 1965; Krakow, 1968) that without DNA template, incubation of RNA polymerase with ATP and UTP or ITP and CTP would synthesize rA:rU or rIrC polymers. These syntheses were characterized by a lag period, a requirement for a higher concentration (0.6 mM) of NTP, and a requirement for Mn^{2+}. Mg^{2+} was not active. No unprimed synthesis was demonstrated with a single NTP. The results from the pyrophosphate release experiments (Table I) show that even with substrate amounts of enzyme no significant phosphodiester bond synthesis could be detected in a 90-min period. (It is expected that for a dissociation constant of 0.015 mM, if the enzyme concentration is 0.038 mM and free NTP concentration is 0.1 mM, the concentration of the bound NTP would be 0.033 mM. If one assumes that all the bound NTP's are dimers, then 0.016 mμmole of $PP_i/\mu l$ should be released, *i.e.*, 0.64 mμmole in a reaction mixture of 40 μl in volume.)

Three techniques have been described in three papers which define binding sites on the RNA polymerase: kinetics (Anthony *et al.*, 1969a), fluorescence quenching (Wu and Goldthwait, 1969), and equilibrium dialysis (this paper). The results are summarized in Table III. We have observed two different NTP binding sites on RNA polymerase with kinetic studies and equilibrium dialysis. With fluorescence studies, however, only the first site was seen. The dissociation constant or apparent K_m of the first site was approximately 0.15 mM while that of the second site was approximately 0.015 mM. With regard to the specificity, the first site binds purine nucleotides. In kinetic studies, the second site which was considered to be the polymerization site showed no selectivity for purines or pyrimidines. On the other hand, in equilibrium dialysis studies, the second site bound primarily purines. The first site has no requirement for divalent metal while the second site does. Rifamycin inhibited the first site but showed no effect on the second.

We postulate that the first site is the initiation site which

TABLE III: Summary of Results Obtained by Kinetics, Fluorescence, and Equilibrium Dialysis Techniques Which Define at Least Two NTP Sites on the RNA Polymerase.

	First Site	Second Site
1. K_s or apparent K_m	0.15 mM (K, F, E)[a]	0.015 mM (K, E)
2. Specificity	Purine (K, F, E)	Purine and pyrimidine (K). purine (E)
3. Divalent metal requirement	No (F, E)	Yes (E)
4. Rifamycin	Inhibition (K, F, E)	No effect (K, E)
5. Postulate	Initiation site	? Polymerization site

[a] K, kinetics; F, fluorescence quenching; E, equilibrium dialysis.

binds the 5′-terminal NTP. This conclusion is based on the similarity of the apparent K_m of initiation and the dissociation constant, on the specificity of the first site to bind purine nucleotides, and on the effect of rifamycin. From the fluorescent studies (Wu and Goldthwait, 1969) it can be concluded that this site is not on the σ protein (Burgess *et al.*, 1969).

The nature of the second site observed by equilibrium dialysis is less certain. Although its dissociation constant is comparable with the apparent K_m of polymerization, it binds primarily purines. It is possible that this site is not the same as the polymerization site observed by kinetic studies. It is also recognized that since the DNA template is not present in the binding studies and its influence on NTP binding is likely to be profound, the interpretation of the binding data is necessarily limited. Kinetic arguments against the presence of an allosteric site have been presented (Anthony *et al.*, 1969a).

One interesting implication of this work is that the preferential initiation of RNA synthesis by purine NTP's is due at least partially to an initiation site on the enzyme.

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Specificity in Self-Assembly of Bacteriophages Qβ and MS2*

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ABSTRACT: Using purified ribonucleic acid and protein components of two serologically and electrophoretically distinct phages Qβ and MS2, phage-like particles were readily assembled from homologous ribonucleic acid and protein as well as from heterologous components. The hybrid particles were antigenically and electrophoretically similar to the homologous particles and to the authentic phages having the same protein component.

The reassembled particles produced from all four ribonucleic acid and protein combinations had similar sedimentation properties in sucrose density gradient. They differed in that the hybrids had no or very low infectivity as compared with the homologous reassembled particles. Under conditions where two phage ribonucleic acids competed for one phage protein, the formation of homologous particles prevailed; whereas when two proteins competed for one ribonucleic acid, Qβ protein was predominantly incorporated into particles with either ribonucleic acid. Particles with mixed protein coat were not detected. Thus the interaction between protein subunits appeared to have absolute species specificity.

Reports from several laboratories show that the RNA coliphages fr (Hohn, 1967), MS2 (Sugiyama *et al.*, 1967), R17 (Roberts and Steitz, 1967), and Qβ (Hung and Overby, 1969) readily reassemble into phage-like particles from RNA and proteins isolated from the respective phages. Hohn (1969) has shown that the protein from fr phage can be induced to form particles by a variety of RNA species. Hiebert *et al.* (1968) found that protein from the plant virus CCMV also could be assembled around RNA from a variety of sources. Particles with mixed-protein coats have been assembled *in vitro* from components of different plant viruses (Wagner and Bancroft, 1968). These results suggest a minimum of specific

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