Studies of Nucleotide Binding to the Ribonucleic Acid Polymerase by a Fluorescence Technique*

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ABSTRACT: Studies with fluorescence spectroscopy showed that *Escherichia coli* ribonucleic acid polymerase had an excitation and emission maximum at 285 and 385 mμ, respectively. The fluorescence intensity at 335 mμ was quenched by the addition of guanosine triphosphate or adenosine triphosphate. On the other hand, cytidine triphosphate and uridine triphosphate had no such effect. The dissociation constants for guanosine triphosphate–enzyme (0.12 mM) and adenosine triphosphate–enzyme (0.16 mM) complexes estimated by fluorimetric titration correlated well with the apparent K_m of initiation reported previously for these two purine nucleoside triphosphates. Divalent metal was not required. Other purine nucleotides yielded dissociation constants with an order nucleoside triphosphate > nucleoside diphosphate > nucleoside monophosphate > deoxynucleoside triphosphate.

Kinetic analysis of the fluorescence data suggested that guanosine triphosphate and adenosine triphosphate competed with each other for a site on the enzyme and that a specific inhibitor of initiation, rifamycin SV, also interacted with the same site (fluorophore) on the enzyme. This site is called the initiation site and it is also present on the enzyme after chromatography on phosphocellulose. The experiments reported here suggest that part of the specificity of initiation with purine nucleotides may be due to the initiation site on the ribonucleic acid polymerase.

The 5′-terminal nucleotide residues of RNA synthesized by the DNA-dependent RNA polymerase are primarily purine nucleotides. The isolation of purine nucleoside tetraphosphates after alkaline hydrolysis of RNA synthesized *in vitro* has provided direct evidence for this (Maitra and Hurwitz, 1965; Maitra *et al.*, 1967; Bremer *et al.*, 1965; Krakow and Horsley, 1967). A similar observation has been made on RNA synthesized *in vivo* (Jorgensen *et al.*, 1969). Indirect evidence for initiation by purine rather than pyrimidine nucleotides is based upon the ability of elevated levels of purine nucleotides to stimulate RNA synthesis (Anthony *et al.*, 1966, 1969a) and also upon the ability of purine nucleotides to give maximum stabilization of the DNA–enzyme–nucleotide complex in the presence of high ionic strength (Anthony *et al.*, 1966). In kinetic studies with *Micrococcus lysodeikticus* DNA, in which GTP is the predominant nucleotide in the 5′-terminal position, an apparent K_m for GTP of approximately 0.15 mM has been interpreted as a K_m for initiation. This is to be compared with an apparent K_m value for the other three nucleotides of 0.015 mM which was considered to represent polymerization (Anthony *et al.*, 1969a).

The mechanism for this specificity of initiation with purine nucleotides is still unknown. The explanation provided by Maitra and Hurwitz (1965) is that the specificity is related to particular base sequences in the DNA. Preferential binding to poly dT has been noted (Jones and Berg, 1966). Thus, the reason for selective copying which results in initiation with purine nucleotides has been postulated to reside in the specificity of the interaction between sites on the DNA and the DNA binding site of the enzyme.

An alternative explanation for some degree of specificity of initiation takes into account a nucleotide binding site on the enzyme. Initiation has been defined in a previous paper (Anthony *et al.*, 1969a) as the formation of the first phosphodiester bond between the 5′-terminal ribonucleoside triphosphate and the subterminal nucleotide. Evidence presented in this paper supports the hypothesis that the site on the enzyme responsible for binding the 5′-terminal nucleoside triphosphate binds purine nucleotides preferentially to pyrimidine nucleotides. In this and the subsequent paper (Wu and Goldthwait, 1969) this site is defined as the initiation site. The evidence provided here is based upon a study of the fluorescent properties of the DNA-dependent RNA polymerase and the ability of nucleotide to decrease this fluorescence. The binding constant for purine nucleoside triphosphates observed by this technique is similar to the apparent K_m of initiation, and evidence is presented that the antibiotic rifamycin which is a specific inhibitor of initiation of RNA synthesis, also interacts with this site on the enzyme.

Experimental Procedures

Biochemicals. L-Tryptophan was purchased from Nutritional Biochemicals Corp. Unlabeled nucleotides and nucleosides were obtained from Sigma Chemical Co., and tritiated nucleoside triphosphates from Schwarz BioResearch, Inc. *M. lysodeikticus* DNA was purchased from the Miles Chemical Co. and after solubilization was filtered through Millipore membranes. Rifamycin SV was a gift from Dr. J. W. Corcoran.

Preparation of RNA Polymerase. Escherichia coli RNA polymerase was prepared from *E. coli* MRE 600 according

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to the procedure of Maitra and Hurwitz (1967) with slight modifications. The procedure involved protamine precipitation and elution, ammonium sulfate precipitation, and DEAE and Bio-Gel chromatography. The enzyme had a specific activity of 4500–6000 units/mg per hr and showed a single peak by sedimentation–velocity ultracentrifugation and from a sedimentation equilibrium study in 0.5 M KCl by the method of Yphantis (1964) a molecular weight of 370,000 was obtained (Wu and Goldthwait, 1969). The enzyme was also prepared by a different procedure which involved chromatography on phosphocellulose (Burgess *et al.*, 1969). The fluorescence data obtained from the enzymes prepared by either method were identical. The enzyme was stored in 30% ammonium sulfate at 4°. Protein concentration was determined by measurements of absorption at 280 mμ using an $E^{1\%}_{1\,cm}$ of 0.67 (Richardson, 1966).

RNA Polymerase Assay. RNA polymerase activity was estimated by the incorporation of [^{3}H]CTP (1400–1800 cpm/mμmole) into trichloroacetic acid insoluble material as described (Anthony *et al.*, 1969a).

Fluorescence Measurement. Fluorescence spectra were measured with an Aminco-Bowman spectrophotofluorometer, or with a spectrophotofluorometer built at this University to specifications slightly modified from those in the literature (Weber, 1956; Teale and Weber, 1957). The same excitation and emission maxima for RNA polymerase (285 and 335 mμ, respectively) were obtained by use of either type of spectrophotofluorometer after correction of the wavelength deviation of the monochromator.

Five-tenths milliliter of the stock enzyme (2–6 mg/ml) was dialyzed against 200 ml of 0.05 M Tris-HCl (pH 7.9) in 0.15 M KCl, 1 mM mercaptoethanol, and 0.1 mM EDTA for 4 hr at 4°. The dialyzed enzyme was diluted to 0.1–0.2 optical density unit at 280 mμ/ml for fluorescence measurements with the Aminco-Bowman spectrophotofluorometer in a synthetic quartz cuvet with a 0.5-cm light path, while only 0.1 ml was required with the Weber-type spectrophotofluorometer. All the measurements were made at 25°.

Fluorometric Titration. The wavelength of excitation and emission were 285 and 335 mμ, respectively. Aliquots (2 μl) of the quencher (nucleotides or rifamycin) were added sequentially to 0.1 ml of the enzyme solution using a 10-μl Hamilton syringe. The quencher was in the same buffer as that of the enzyme solution. Fluorescence intensity was measured at 25° after each addition and thorough mixing. The time required for each fluorometric titration was approximately 15 min. Optical density at the excitation wavelength was measured before and after each titration in order to correct for the inner filter effect (see below).

Correction of Fluorescence Data. Calibration of the monochromator was done with a mercury lamp of known emission wavelength. Instability of the light source was compensated for by the ratio recorder. No correction has been made for the variation with wavelength in the sensitivity of the detection system.

The decrease in fluorescence due to the dilution of the fluorescent substance caused by the additions was estimated by adding the same amount of the buffer solution without quencher. This was done for each experiment and the appropriate corrections were made.

The inner filter effect due to absorption of the exciting radiation by the quencher and protein itself in the solution

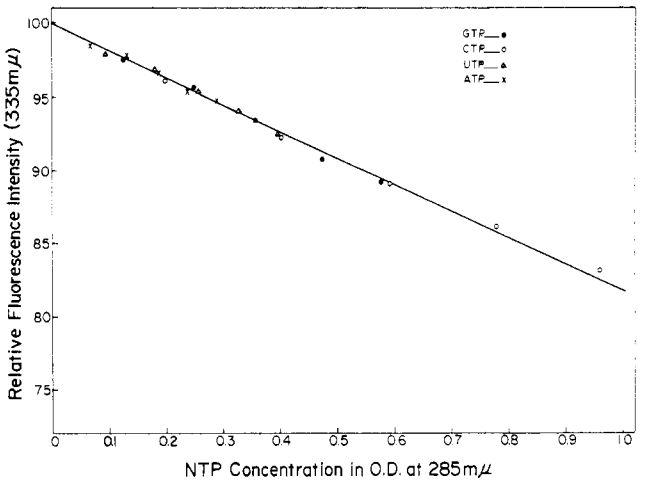


FIGURE 1: Fluorometric titration of L-tryptophan with various nucleoside triphosphates. All titrations were performed with 10^{-4} M L-tryptophan in 0.05 M Tris-HCl (pH 7.9) in 0.15 M KCl, 1 mM mercaptoethanol, and 0.1 mM EDTA at 25°. The fluorescence was measured with the Weber-type spectrophotofluorometer. The wavelengths of excitation and emission were 285 and 335 mμ. Nucleoside triphosphate concentrations varied from 0.04 to 0.2 mM. The data have been corrected for dilution effect. Titration with GTP, ●—●; ATP, x—x; CTP, ○—○; UTP, △—△.

was corrected experimentally as follows. Titrations of 0.1 mM tryptophan with various nucleoside triphosphates in concentrations ranging from 0.04 to 0.2 mM were performed with excitation and emission wavelengths at 285 and 335 mμ, respectively. When the relative fluorescence intensity after correction for dilution was plotted against the concentration of nucleoside triphosphate represented by optical density at the excitation wavelength, all the points fell on a straight line as shown in Figure 1. This indicates that in this range of concentrations there is no specific interaction between any of the four NTP's[1] and tryptophan. The decrease in fluorescence intensity must be the result of the inner filter effect since it is a function of optical density. Thus, this plot was used as a standard correction curve for the inner filter effect at various optical densities of the measured solution. The experimental correction of the inner filter effect was checked by a theoretical approach when the Aminco-Bowman spectrophotofluorometer was used. With the slit wide open, intensities of fluorescence were corrected for the inner filter effect by using the relationship (Brand and Witholt, 1967)

$$I_{cor} = I_{obsd} \times \frac{2.3 \times (OD)^{1\,cm}_{\lambda e} \times l}{1-10^{-(OD)^{1\,cm}_{\lambda e} \times l}}$$

where $(OD)^{1\,cm}_{\lambda e}$ refers to the optical density of the solution measured at the excitation wavelength with a light path equal to 1 cm, and l is the light path of the cuvet in centimeters. That the corrections are not a source of error is suggested by the consistency of the data obtained with the Aminco-Bowman and with the Weber apparatus.

[1] Abbreviations used are: NTP, nucleoside triphosphate; RM, rifamycin SV.

Theory

One Quencher System. A general mechanism which satisfies the data presented below involved interaction between a fluorescent molecule, E (enzyme), and a quencher, Q. The system can be formulated as

$$[E] + [Q] \underset{}{\overset{K}{\rightleftharpoons}} [EQ] \xrightarrow{k} \Delta f$$

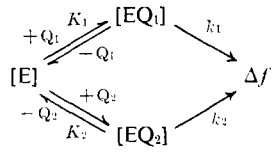
where K is the dissociation constant of the binary complex, EQ, Δf is the measured quenching of the fluorescence intensity (*i.e.*, $I_0 - I$, where I_0 is the fluorescence intensity in the absence of the quencher and I is the fluorescence intensity when the quencher is at a specific concentration), and k the quenching constant. With the relationship $[E_0] = [E] + [EQ]$, $K = [E][Q]/[EQ]$, and $\Delta f = k[EQ]$, where $[E_0]$ and $[E]$ refer to the total and free concentrations of the fluorescent molecules and [Q] and [EQ] refer to concentrations of free quencher and the encounter complex, an expression analogous to the Lineweaver–Burk equation can be derived in which ΔF is

$$\frac{1}{\Delta f} = \frac{1}{\Delta F} + \frac{K}{\Delta F}\frac{1}{[Q]} \tag{1}$$

the fluorescence quenching when the fluorescent molecule is completely complexed with the quencher.

Two Quencher System. The question whether two quenchers do or do not appear competitive with each other (*i.e.*, presumably react at the same site) can be answered if certain data are available.

Case I. Competitive binding of two quenchers to a single site in the fluorescence molecule. No ternary complex exists in this case. This type of system can be represented by



where the subscripts 1 and 2 refer to the first and second quenchers.

Introduction of the dissociation constants $K_1 = [E][Q_1]/[EQ_1]$ and $K_2 = [E][Q_2]/[EQ_2]$ into the equation for the total concentration of the fluorescent molecules, $[E_0] = [E] + [EQ_1] + [EQ_2]$, yields

$$[E_0] = [E]\left(1 + \frac{[Q_1]}{K_1} + \frac{[Q_2]}{K_2}\right) \tag{2}$$

Assuming that Δf_1 and Δf_2 are additive the quenching of the system, $\Delta f = k_1[EQ_1] + k_2[EQ_2]$ can be expressed as

$$\Delta f = \frac{k_1}{K_1}[E][Q_1] + \frac{k_2}{K_2}[E][Q_2] \tag{3}$$

Substitution of [E] in eq 3 by eq 2 gives

$$\Delta f = \frac{[E_0]}{1 + \frac{[Q_1]}{K_1} + \frac{[Q_2]}{K_2}}\left(\frac{k_1}{K_1}[Q_1] + \frac{k_2}{K_2}[Q_2]\right) \tag{4}$$

With $\Delta F_1 = k_1[E_0]$ when $[Q_1] \to \infty$ and $\Delta F_2 = k_2[E_0]$ when $[Q_2] \to \infty$, eq 4 may be written as

$$\Delta f = \frac{\frac{\Delta F_1}{K_1}[Q_1] + \frac{\Delta F_2}{K_2}[Q_2]}{1 + \frac{[Q_1]}{K_1} + \frac{[Q_2]}{K_2}} \tag{5}$$

Through some algebraic manipulation, eq 5 may be rewritten as

$$\Delta f = \Delta F_1 + \frac{\frac{\Delta F_2}{K_2}[Q_2] - \Delta F_1\left(1 + \frac{[Q_2]}{K_2}\right)}{\frac{[Q_1]}{K_1} + \left(1 + \frac{[Q_2]}{K_2}\right)} \tag{6}$$

or

$$\Delta F_1 - \Delta f = \frac{\Delta F_1\left(1 + \frac{[Q_2]}{K_2}\right) - \Delta F_2\frac{[Q_2]}{K_2}}{\frac{[Q_1]}{K_1} + \left(1 + \frac{[Q_2]}{K_2}\right)} \tag{7}$$

If reciprocals are taken, the result is

$$\frac{1}{\Delta F_1 - \Delta f} = \frac{\frac{1}{K_1}}{\Delta F_1\left(1 + \frac{[Q_2]}{K_2}\right) - \Delta F_2\frac{[Q_2]}{K_2}}[Q_1] + \frac{1 + \frac{[Q_2]}{K_2}}{\Delta F_1\left(1 + \frac{[Q_2]}{K_2}\right) - \Delta F_2\frac{[Q_2]}{K_2}} \tag{8}$$

In the case where the concentration of one quencher is held constant, for instance, $[Q_2]$ = constant, then a plot of $1/(\Delta F_1 - \Delta f)$ *vs.* $[Q_1]$ should yield a straight line with

$$\text{slope} = \frac{\frac{1}{K_1}}{\Delta F_1\left(1 + \frac{[Q_2]}{K_2}\right) - \Delta F_2\frac{[Q_2]}{K_2}} \tag{9}$$

intercept on the ordinate, Iy =

$$\frac{1 + \frac{[Q_2]}{K_2}}{\Delta F_1\left(1 + \frac{[Q_2]}{K_2}\right) - \Delta F_2\frac{[Q_2]}{K_2}} \tag{10}$$

and intercept on the abscissa, Ix =

$$K_1 + \frac{K_1}{K_2}[Q_2] \tag{11}$$

From eq 11 it is seen that Ix increases with raising the concentration of Q_2, and when $[Q_2] = 0$,

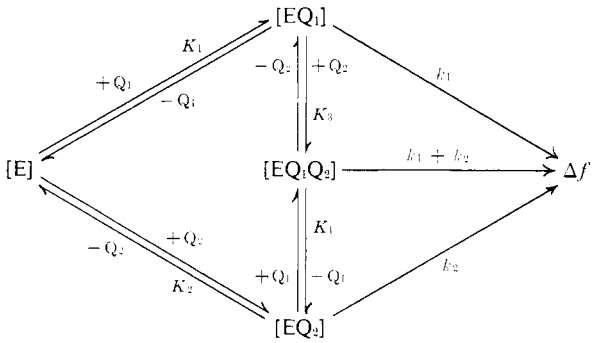
$$Ix = K_1 \tag{12}$$

and also

$$Iy = \frac{1}{\Delta F_1} \tag{13}$$

This type of plotting is made possible since ΔF_1 can be obtained experimentally by titration with Q_1 alone in the absence of Q_2. However, there are two limitations. Firstly, ΔF_1 should be greater than Δf for a continuous plot, hence Q_1 must be so selected that $\Delta F_1 > \Delta F_2$. Secondly, since $[Q_{2_0}] = [Q_2] + [EQ_2]$ and experimentally we fix $[Q_{2_0}]$ and not $[Q_2]$, the relationship $[Q_2] \gg [EQ_2]$ should be held, so that $[Q_2] \simeq [Q_{2_0}] =$ constant. This implies that only when $[Q_{2_0}] \gg [E_0]$, can this type of plot be made meaningful,

CASE II. Noncompetitive binding of two quenchers to separate sites in the fluorescent molecule. In this case a ternary complex is formed and the following reaction scheme may be proposed for this system.



where EQ_1Q_2 = ternary complex, $K_3 = [EQ_1][Q_2]/[EQ_1Q_2]$, and $K_4 = [EQ_2][Q_1]/[EQ_1Q_2]$, and the definitions of other symbols are the same as those described in case I. By use of the next three basic relationships

$$[E_0] = [E] + [EQ_1] + [EQ_2] + [EQ_1Q_2] \tag{14}$$

$$\Delta f = k_1[EQ_1] + k_2[EQ_2] + (k_1 + k_2)[EQ_1Q_2] \tag{15}$$

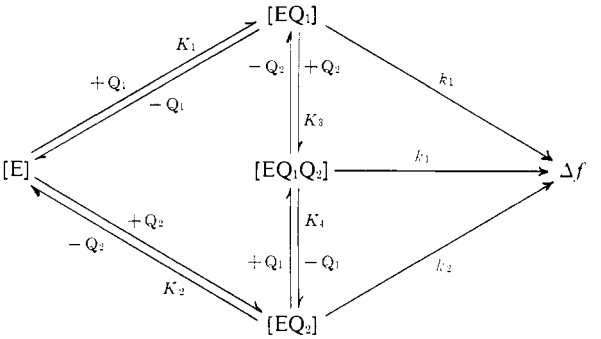
$$K_1K_3 = K_2K_4 \tag{16}$$

an equation equivalent to eq 8 can be derived through similar manipulations used in case I.

$$\frac{1}{\Delta F_1 - \Delta f} = \frac{\left(1 + \frac{[Q_2]}{K_2}\right) + \left(\frac{1}{K_1} + \frac{[Q_2]}{K_1K_3}\right)[Q_1]}{\Delta F_1 + (\Delta F_1 - \Delta F_2)\frac{[Q_2]}{K_2} - \frac{\Delta F_2[Q_1]}{K_1K_3}[Q_1]} \tag{17}$$

It is clear from the above equation that a plot of $1/(\Delta F_1 - \Delta f)$ *vs.* $[Q_1]$ will not appear linear.

CASE III. Two quenchers binding to two sites which are close enough to quench the same fluorophore. Assume the quenching is competitive in such a manner that in the ternary complex only the first quencher is effective and the second is completely blocked. In this case the reaction scheme will be



An equation equivalent to eq 8 or 16 is obtained by a similar derivation

$$\frac{1}{\Delta F_1 - \Delta f} = \frac{\frac{1}{K_1} + \frac{[Q_2]}{K_1K_3}}{\Delta F_1 + (\Delta F_1 - \Delta F_2)\frac{[Q_2]}{K_2}}[Q_1] + \frac{1 + \frac{[Q_2]}{K_2}}{\Delta F_1 + (\Delta F_1 - \Delta F_2)\frac{[Q_2]}{K_2}} \tag{18}$$

A plot of $1/(\Delta F_1 - \Delta f)$ *vs.* $[Q_1]$ will again yield a straight line which has the same Iy but a different slope from that in case I. The intercept on the base line will be

$$Ix = \frac{1 + \frac{[Q_2]}{K_2}}{\frac{1}{K_1} + \frac{[Q_2]}{K_1K_3}} \tag{19}$$

Therefore cases I and III can be differentiated by the Ix value obtained. However, if $[Q_2] \ll K_3$, then $1/K_1 \gg [Q_2]/K_1K_3$ and $Ix \simeq K_1 + K_1/K_2[Q_2]$. Under these conditions, cases I and III are indistinguishable.

Results

Fluorescence Properties of E. coli RNA Polymerase. Figure 2 shows the fluorescence excitation and emission spectra of *E. coli* RNA polymerase in 0.05 M Tris-HCl (pH 7.9) with 0.15 M KCl, 1 mM mercaptoethanol, and 0.1 mM EDTA. This was measured at 25° with the Aminco-Bowman spectrophotofluorometer and a 0.5-cm light-path cuvet. The excitation and emission maxima were at 285 and 335 mμ, respectively. The addition of 8 mM $MgCl_2$ or 2 mM $MnCl_2$ produced no detectable change in either the excitation or the emission spectrum. The phosphocellulose enzyme prepared by the method of Burgess *et al.* (1969) has the same fluorescence excitation and emission spectra as that shown in Figure 2.

Titration of RNA Polymerase with Nucleoside Triphosphates. The effect of different concentrations of nucleotides on the

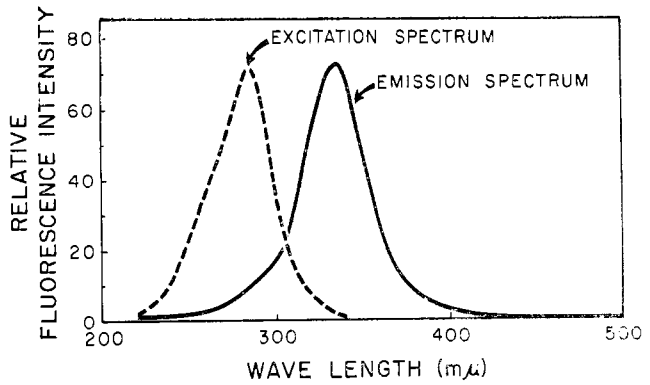


FIGURE 2: Fluorescence excitation and emission spectra of *E. coli* RNA polymerase. Solutions contained 1 μM *E. coli* RNA polymerase, 0.05 M Tris-HCl (pH 7.9), 0.15 M KCl, 1 mM mercaptoethanol, and 0.1 mM EDTA. Fluorescence was measured with the Aminco-Bowman spectrophotofluorometer at 25° using a 0.5-mm synthetic quartz cuvet. The excitation and emission wavelengths were 285 and 335 mμ for emission and excitation spectra, respectively.

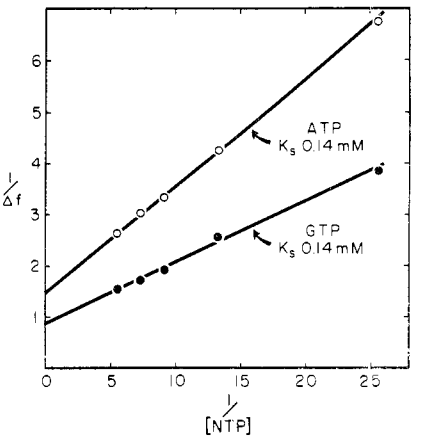


FIGURE 4: The effect of different concentrations of GTP and ATP on the fluorescence of RNA polymerase. Data taken from Figure 3, plotted according to eq 1. The quenching of the fluorescence of Δf is the intensity observed in the absence of nucleoside triphosphates minus the intensity observed in the presence of nucleoside triphosphates minus the intensity observed in the presence of nucleoside triphosphates. Titration with GTP, ●—●; with ATP, ○—○.

fluorescence intensity of RNA polymerase is shown in Figure 3. The fluorescence intensity at 335 mμ decreases with no apparent shift in the wavelength maximum on the addition of GTP or ATP. CTP and UTP have no such effect. Similar findings have been observed with the phosphocellulose enzyme. This titration curve was done in the absence of divalent metal and in the presence of EDTA. When done in the presence of 10 mM $MgCl_2$ or 2 mM $MnCl_2$, the titration is the same. A plot of $1/\Delta f$ *vs.* 1/[NTP] according to eq 1 (Figure 4) yields an apparent dissociation constant of 0.14 mM for both GTP and ATP. In a series of experiments, values in a range between 0.12 and 0.17 mM were obtained. The value observed by this technique is similar to the apparent K_m for initiation obtained by kinetic studies (Anthony *et al.*, 1969a). When *p*-mercuriphenylsulfonate at 10^{-3} M was added, a noncompetitive type of inhibition of the GTP–enzyme interaction was observed.

Effect of Other Purine Nucleotides and Nucleosides. To determine whether other purine derivatives act in a manner similar to GTP and ATP, various purine compounds were tested with the RNA polymerase. First it was noted that the extent of maximum quenching decreases in the order of GTP > ATP > ITP and NTP > NDP > NMP > dNTP. Adenine,

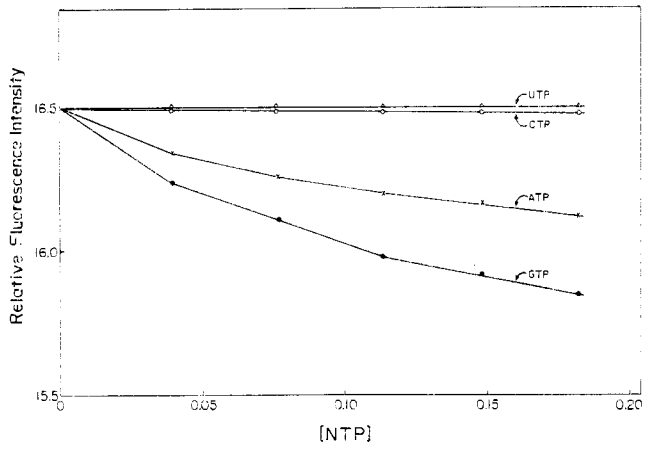


FIGURE 3: Fluorometric titration of *E. coli* RNA polymerase with nucleoside triphosphates. Studies were performed with enzyme (0.5 μM) in 0 05 M Tris-HCl (pH 7.9), 0.15 M KCl, 1 mM mercaptoethanol, and 0.1 mM EDTA at 25° with the Weber-type spectrophotofluorometer. The wavelengths of excitation and emission were 285 and 335 mμ. The data were corrected for both dilution and the inner filter effect. Titration with GTP, ●—●; ATP, ×—×; CTP, ○—○; and UTP, △—△.

TABLE I: Dissociation Constants for Binary Complexes of RNA Polymerase and Various Purine Derivatives.[a]

Purine Derivatives	Dissocn Constant (mM)
GTP	0.12
GDP	0.49
GMP	0.80
dGTP	1.87
ATP	0.16
ADP	0.35
AMP	0.57
dATP	*b*
Adenosine	*b*
Adenine	*b*
ITP	0.133

[a] Data were obtained by titration of RNA polymerase with various purine derivatives. Solutions, pH, temperature, excitation, and emission wavelengths were the same as Figure 3. The inner filter affect was corrected for each purine derivative using Figure 1. The dissociation constants were obtained with plots according to eq 1. [b] No effect on fluorescence at concentrations up to 0.2 mM.

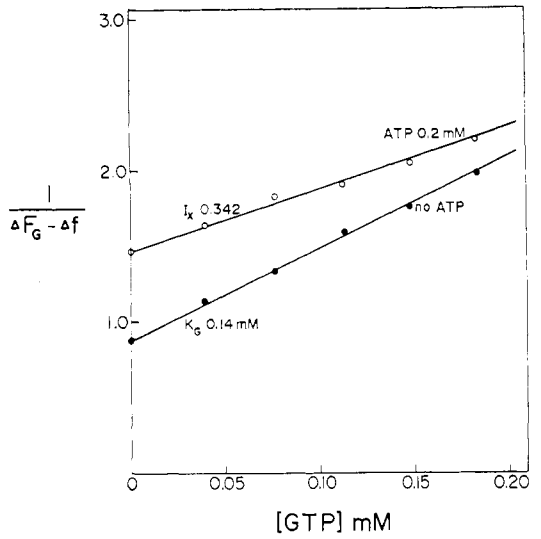


FIGURE 5: Effect of GTP on the fluorescence intensity of RNA polymerase in the presence and absence of ATP. Solutions, pH, temperature, excitation, and emission wavelengths were the same as Figure 3. The titration data were then plotted according to eq 8. ΔF_G was obtained from Figure 4. Titration with GTP in the absence of ATP (●—●) and in the presence of 0.2 mM ATP (○—○).

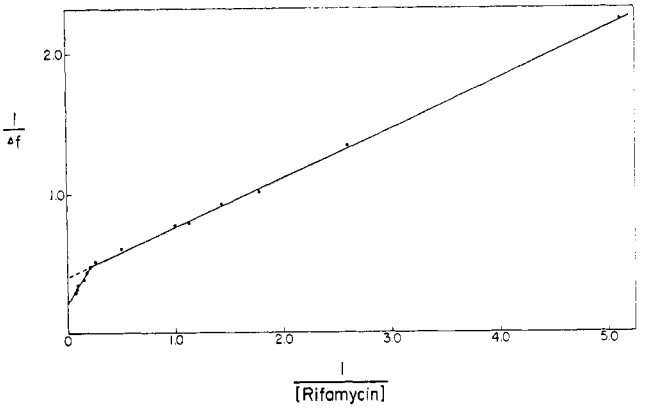


FIGURE 6: Fluorometric titration of RNA polymerase with rifamycin SV. Solutions, pH, temperature, excitation, and emission wavelengths were the same as in Figure 3. The inner filter effect due to rifamycin was corrected with an experimental approach similar to that in Figure 1. The reciprocal of the difference in fluorescence intensity in the absence and presence of rifamycin is plotted as a function of the reciprocal of rifamycin concentration according to eq 1.

adenosine, and dATP have no detectable effect on the fluorescence. Secondly, the dissociation constants for the various purine derivatives were determined and are presented in Table I. In this case the relationship NTP > NDP > NMP > dNTP also holds. ITP has a value similar to the dissociation constants of GTP and ATP.

Competition between GTP and ATP. $\Delta F_G = 1.15$ and $\Delta F_A = 0.69$ were obtained from Figure 4. If the concentration of ATP is kept constant at 0.2 mM, and the enzyme concentration used is 0.5×10^{-6} M, the two limiting conditions for eq 8 will be satisfied, *i.e.*, $\Delta F_G > \Delta F_A$ and $[E]_0 \ll [ATP]_0$. It is shown in Figure 5 that a plot of $1/(\Delta F_G - \Delta f)$ *vs.* [GTP] gives a straight line. The value of Ix determined experimentally, Ix_{obsd}, is 0.342 and is in accord with that calculated from eq 11 (based on the previously estimated K_G and K_A values), which is 0.34. The data presented suggest that GTP and ATP are mutually competitive and probably react at the same site on the enzyme. CTP or UTP at 0.2 mM did not change the dissociation constant of either GTP or ATP, and hence do not bind at the same site.

Interactions of Rifamycin with RNA Polymerase. Data obtained in a titration of RNA polymerase with rifamycin SV are given in Figure 6. When the concentrations of rifamycin SV are in the range from 0.2 to 15 μM, a plot of $1/\Delta f$ *vs.* 1/[RM] yields a biphasic line. This might be due to two different types of rifamycin binding sites on the enzyme. However, at concentrations of rifamycin lower than 1 μM, the plot was linear within experimental error. From this data a K_{RM} of 0.9 μM and a ΔF_{RM} of 2.50 were calculated.

Effect of GTP on RM–RNA Polymerase Interaction. Since ΔF_{RM} is greater than ΔF_G or ΔF_A, it also satisfies the prescribed conditions to plot $1/(\Delta F_{RM} - \Delta f)$ against [RM]. As shown in Figure 7, when either GTP or ATP is held constant at 0.2 mM a straight line according to eq 8 or 18 was obtained. The experimental values of $(Ix)_G$ was 2.04 and $(Ix)_A$ was 2.23. These were similar to the calculated value (eq 11) of $Ix = 2.12$. This suggests that rifamycin either binds at the same site with purine nucleotides (case I) or binds to a different site but quenches the same fluorophore in the enzyme as do the purine nucleotides (case III).

Effect of Rifamycin on the Kinetics of the RNA Polymerase Reaction. As discussed previously (Anthony *et al.*, 1969a) when *M. lysodeikticus* DNA was used as a primer, and the concentration of ATP, CTP, and UTP was high while GTP was varied, an apparent K_m of initiation (0.15 mM) was ob-

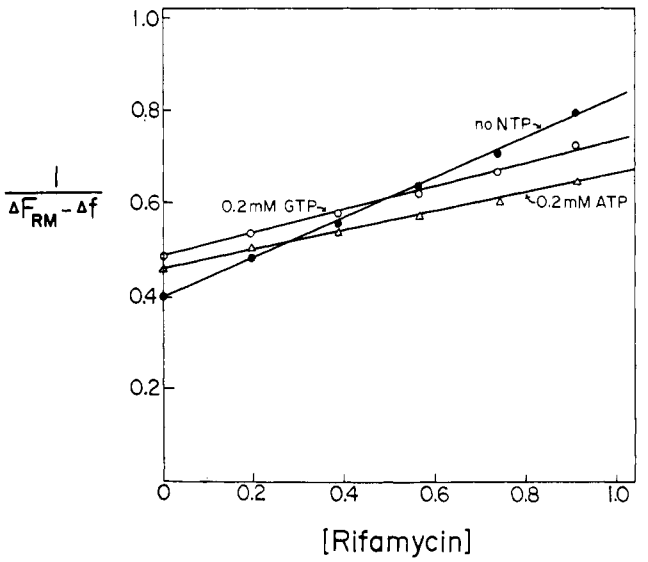


FIGURE 7: Effect of rifamycin on the fluorescence intensity of RNA polymerase in the presence and absence of purine nucleoside triphosphates. Solution, pH, temperature, excitation, and emission wavelengths were the same as in Figure 3. The titration data were plotted according to Eq 8. ΔF_{RM}, which was the maximum quenching when the fluorescent molecule is completely complexed with rifamycin, was obtained from Figure 6. Titration with rifamycin SV in the absence of purine nucleoside triphosphate (●—●), in the presence of 0.2 mM GTP (○—○), and in the presence of 0.2 mM ATP (△—△).

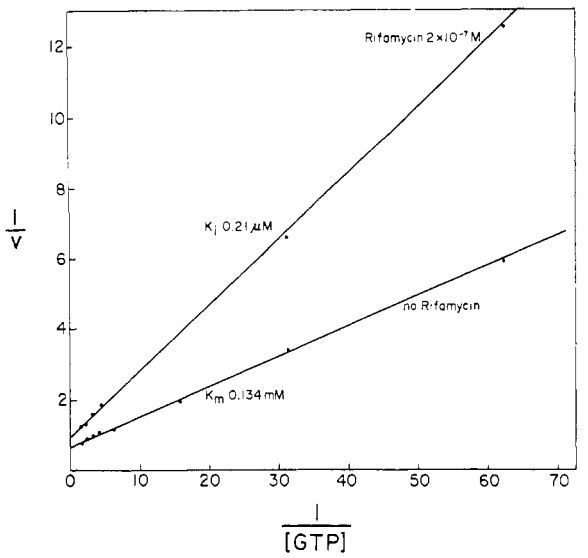


FIGURE 8: Effect of rifamycin SV on the kinetics of initiation. Incubation mixtures, 0.5 ml in volume, contained 50 mM Tris-HCl (pH 7.9), 2 mM $MnCl_2$, 8 mM $MgCl_2$, 0.15 M KCl, 0.54 mM mercaptoethanol, 25 μg of *M. lysodeikticus* DNA, and 15 μg of enzyme. The GTP concentration is expressed as millimolar and was varied from 0.01 to 0.4 mM. The concentration of ATP, UTP, and [^{3}H]CTP (1300 cpm/mμmole) was fixed at 0.4 mM. Rifamycin was either absent or present at 0.2 mM. Following a 10-min preincubation at 0°, reactions were started by addition of the enzyme. Incubation for 5 min at 37° was terminated by addition of a 0.3-ml aliquot to 5 ml of cold 5% trichloroacetic acid in a Millipore filter apparatus. Precipitates were washed with 5% trichloroacetic acid and counted in a scintillation system.

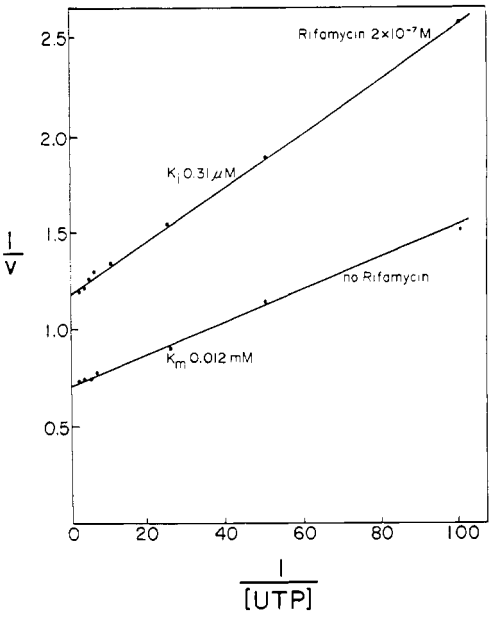


FIGURE 9: Effect of rifamycin SV on the kinetics of polymerization. The precincubation and incubation were similar to those noted under Figure 8 except that the concentration of UTP was varied and the concentration of ATP, GTP, and [^{3}H]CTP was kept constant at 0.4 mM.

served. Under these conditions the RNA chains are initiated primarily with GTP. On the other hand, when the UTP concentration was varied and the concentration of GTP, ATP, and CTP was high, an apparent K_m of polymerization (0.015 mM) was observed. The effects of rifamycin SV on these two systems are presented in Figures 8 and 9. A mixed-type inhibition was found with varying concentrations of GTP (Figure 8), while a noncompetitive inhibition was observed when the UTP concentration was varied (Figure 9). The K_i values for rifamycin SV were 0.21 μM with GTP and 0.31 μM with UTP.

Discussion

By a fluorescent technique, it has been possible to define a binding site on the RNA polymerase for purine nucleotides. The fluorescence of RNA polymerase is largely due to the tryptophan residues, as characterized by excitation and emission spectra. The binding of purine nucleotides to RNA polymerase results in a partial quenching of tryptophan fluorescence which is interpreted as a specific interaction of the purine nucleotides with one or several tryptophanyl residues in or near the initiation site of the enzyme. This is based on three lines of evidence. First, the value of the dissociation constants for purine nucleoside triphosphates calculated from the fluorescence titration data is the same as that of the apparent K_m of initiation obtained from the kinetic studies on the incorporation of nucleotides. Second, the selective interaction observed by fluorescence quenching between the RNA polymerase and GTP or ATP compared with the lack of interaction with CTP and UTP correlates with the direct and indirect evidence for involvement of purine rather than pyrimidine nucleotides in initiation noted earlier. The order of affinity of purine nucleotides for the RNA polymerase was found in the fluorescence experiments to be GTP > GDP > GMP > dGTP and ATP > ADP > AMP > dATP. There is no direct evidence for or against the sence of purine nucleoside diphosphates in the 5′-terminal position. One experiment provides some indirect evidence for this.

With calf thymus DNA, when the concentration of the four nucleoside triphosphates is varied, a double-reciprocal plot of incorporation versus nucleotide concentration is curvilinear. A high concentration of ATP with the other three nucleotides varied produces linear kinetics and this has been interpreted to be due to its effect on initiation. ADP at a high concentration, with the four nucleoside triphosphates varied, also eliminates the curvilinearity, presumably due to an effect on initiation (Anthony *et al.*, 1969a). The third piece of evidence which linked the fluorescent changes to the initiation binding site is related to the effect of rifamycin. Rifamycin SV is a specific inhibitor of the initiation of RNA synthesis and has no effect on polymerization (Sippel and Hartmann, 1968). Analysis of the fluorescence data suggested that rifamycin competes with the GTP and ATP binding to the RNA polymerase.

Thus the experiments reported above suggest the interesting possibility that some specificity of initiation may reside at a nucleotide binding site on the enzyme as well as in the enzyme–DNA binding site and the DNA base sequence. A preferential enzyme interaction of purine compared with pyrimidine nucleoside triphosphates has been observed by several techniques. First, the RNA polymerase binds to both DNA and tRNA

and high levels of nucleotides are able to dissociate the complexes; purine nucleoside triphosphates are more effective than pyrimidine nucleoside triphosphates (Anthony *et al.*, 1969b). Second, it was observed that the enzyme was inactivated by elevated levels of purine nucleoside triphosphates much more readily than by pyrimidine nucleoside triphosphates (Anthony *et al.*, 1969b). Also GTP at 10 mM caused an aggregation of the RNA polymerase while comparable levels of CTP did not (C. W. Wu, unpublished observations). Finally preincubation of the RNA polymerase with a single nucleoside triphosphate in the absence of DNA could protect the enzyme from alteration by heat as measured by the ability of the enzyme to bind to DNA. GTP was the most effective nucleotide (Stead and Jones, 1967).

The mechanism of action of rifamycin on RNA polymerase has been studied by several workers. Sippel and Hartman (1968) made several pertinent observations. The antibiotic had no effect on RNA synthesis when added after the start of the reaction; the effect of the antibiotic on RNA synthesis was independent of the base composition of the DNA template; and the inhibition by the antibiotic was reduced by preincubation of the enzyme–DNA complex with purine nucleotides. These observations suggested that the antibiotic affected initiation of RNA synthesis (Wehrli *et al.*, 1968b). The isolation of a mutant of *E. coli* with a rifamycin-resistant RNA polymerase (Tocchini-Valentini *et al.*, 1968; Ezekiel and Hutchins, 1968) implied that rifamycin interacts with the enzyme. Binding of the antibiotic to RNA polymerase isolated from rifamycin-sensitive *E. coli*, but not to the enzyme from rifamycin-resistant cells has been observed (Wehrli *et al.*, 1968a). Our present experiments agree with these observations and also suggest that rifamycin may bind to a point which is in or close to the binding site for the 5′-terminal nucleoside triphosphate. We shall call this the initiation site.[2] This site is present on the phosphocellulose enzyme in our experiments. Whether rifamycin binds to the same site as the purine nucleoside triphosphates (case I) or whether it binds to a different site but close enough to quench the fluorescence of the same tryptophan residue (case III) is difficult to answer by our present data. Assuming case III conditions are valid, a K_3 value obtained is indeed much higher than $[Q_3]$ (0.2 mM). According to the argument we have presented for eq 19, we are not able to differentiate case I and case II under these circumstances.

There are several differences between the fluorescence system we have studied and the system involving RNA synthesis by the enzyme: (a) no DNA is present in the fluorescence system but it is required for RNA synthesis. There is a definite effect of the template DNA on initiation. The predominance of either GTP or ATP in the 5′ termini of the RNA chains is dependent upon the DNA template (Maitra *et al.*, 1967). Whether this is due entirely to a specific initiation site on the DNA template has yet to be assessed. (b) No metals such as Mg^{2+} or Mn^{2+} are required for the effect of nucleotides in the fluorescence system. This is contrary to the fact that these metals are necessary for enzymatic activity. If there are nucleotide binding sites for the 5′-terminal and subterminal nucleotides, metal may be required only for binding of the subterminal nucleotide. There may be a tightly bound metal at the 5′-terminal nucleotide site. Metal may also be required for the formation of the phosphodiester bond. It is recognized that trace amounts of metals may exist in our system even in the presence of EDTA. (c) Rifamycin exhibits a mixed type of inhibition on the initiation kinetics with GTP and *M. lysodeikticus* DNA while competitive binding was observed with fluorescence quenching. Furthermore the observed binding constants for rifamycin differ. One possible explanation is that rifamycin and GTP bind to two different sites but close enough to quench the same fluorophore as described in case III. In this circumstance, an apparent competitive type of kinetics would be observed in the fluorescent studies according to eq 18. On the other hand, the existence of a ternary complex might lead to a mixed-type inhibition in the enzymatic study. This assumption is compatible with our present data although the kinetic studies are only suggestive and give no definite proof. Another reason for the difference may be that in the fluorescence system, rifamycin actually competes with GTP at the same site, but when DNA is present, as in the enzymatic system, the nucleotide binding by the enzyme may be so altered that a ternary complex is formed which causes the mixed-type inhibition observed.

It was found recently that the DNA-dependent RNA polymerase from *E. coli* can be further separated into a basic polymerase (phosphocellulose enzyme) and a protein factor (Sigma) by chromatography on a phosphocellulose column (Burgess *et al.*, 1969). The phosphocellulose enzyme is active with calf thymus DNA as template, but fails to transcribe phage T4 DNA appreciably. Addition of the protein factor to the phosphocellulose enzyme fully restores the template activity of T4 DNA. There is evidence that indicates the Sigma protein markedly increases the number of RNA chains initiated. This suggests that Sigma acts at the level of initiation. Our results of fluorescence studies of the nucleotide–enzyme interaction demonstrates no difference between the phosphocellulose enzyme and the enzyme prepared by the ordinary method without phosphocellulose column chromatography. This implies that the phosphocellulose enzyme possesses the nucleotide initiation site and is in accord with the fact that the phosphocellulose enzyme can by itself initiate the synthesis of RNA chains. This fluorescence observation agrees with those of Mauro *et al.* (1969) which indicated that the sensitivity to rifamycin does not reside on the sigma factor.

Finally, results obtained by equilibrium dialysis and reported in the following paper (Wu and Goldthwait, 1969) indicate that only one purine binding site, with a K_s of approximately 0.15 mM and which is affected by rifamycin, exists per molecule of RNA polymerase with a molecular weight of 370,000.

[2] The initiation site binds one nucleoside triphosphate per enzyme molecule of 370,000 molecular weight as is shown in the following paper (Wu and Goldthwait, 1969). The process of initiation requires the synthesis of the first phosphodiester bond and therefore the binding of two nucleoside triphosphates to the enzyme—one, to the initiation site and the second to a different site (Anthony *et al.*, 1969a).

References

Anthony, D. D., Wu, C. W., and Goldthwait, D. A. (1969a), *Biochemistry 8*, 246.

Anthony, D. D., Zeszotek, E., and Goldthwait, D. A. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1026.

Anthony, D. D., Zeszotek, E., and Goldthwait, D. A.

(1969b), *Biochim. Biophys. Acta 174*, 458.
Brand, L., and Witholt, B. (1967), *Methods Enzymol. 11*, 776.
Bremer, G., Konrad, M. W., Gaines, K., and Stent, G. S. (1965), *J. Mol. Biol. 13*, 540.
Burgess, R. R., Travers, A. A., Dunn, J. J., and Bautz, E. K. F. (1969), *Nature 221*, 43.
Ezekiel, D. H., and Hutchins, J. E. (1968), *Nature 220*, 276.
Jones, O. W., and Berg, P. (1966), *J. Mol. Biol. 22*, 199.
Jorgensen, S. E., Buch, L. B., and Nierlich, D. P. (1969), *Science 164*, 1067.
Krakow, J., and Horsley, W. (1967), *J. Biol. Chem. 242*, 4796.
Maitra, U., and Hurwitz, J. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 815.
Maitra, U., and Hurwitz, J. (1967), *J. Biol. Chem. 242*, 4897.
Maitra, U., Nakata, Y., and Hurwitz, J. (1967), *J. Biol. Chem. 242*, 4908.
Mauro, E. di., Snyder, L., Marino, P., Lamberti, A., Coppo, A., and Tocchini-Valentini, G. P. (1969), *Nature 222*, 533.
Richardson, J. P. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 1616.
Sippel, A., and Hartmann, G. (1968), *Biochim. Biophys. Acta 157*, 218.
Stead, N. W., and Jones, O. W., Jr. (1967), *Biochim. Biophys. Acta 145*, 679.
Teale, F. W. J., and Weber, G. (1957), *Biochem. J. 65*, 476.
Tocchini-Valentini, G. P., Marino, P., and Colvill, A. J. (1968), *Nature 220*, 275.
Weber, G. (1956), *J. Opt. Soc. Am. 46*, 962.
Wehrli, W., Knüsel, F., Schmid, K., and Staehelin, M. (1968a), *Proc. Natl. Acad. Sci. U. S. 61*, 667.
Wehrli, W., Knüsel, F., and Staehelin, M. (1968b), *Biochem. Biophys. Res. Commun. 22*, 284.
Wu, C. W., and Goldthwait, D. A. (1969), *Biochemistry 8*, 4458.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

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

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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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