*INITIATION BY THE DNA-DEPENDENT RNA POLYMERASE**

By Donald D. Anthony,† Eileen Zeszotek, and David A. Goldthwait‡

DEPARTMENT OF BIOCHEMISTRY, WESTERN RESERVE UNIVERSITY, CLEVELAND, OHIO

Communicated by Harland G. Wood, July 25, 1966

This communication presents evidence which indicates that RNA synthesis by the DNA-dependent RNA polymerase occurs in three steps:

(1) Association: DNA + Enzyme $\rightleftharpoons$ DNA-enzyme.

(2) Initiation: DNA-enzyme + purine nucleotide $\longrightarrow$ $\left[\begin{matrix}\text{DNA-enzyme-purine} \\ \text{nucleotide}\end{matrix}\right]$.

(3) Polymerization: $\left[\begin{matrix}\text{DNA-enzyme-purine} \\ \text{nucleotide}\end{matrix}\right]$ + NTP $\longrightarrow$ DNA-enzyme-oligoribonucleotide + PP_i.

The formation of the DNA-enzyme complex, step 1, can be inhibited by high ionic strength. When initiation occurs, a different DNA-enzyme complex is formed in the presence of purine nucleoside triphosphates which is not as easily dissociated by high ionic strength. The initiation complex can also be detected by a specially devised membrane assay. The exact nature of this complex is not established, but for its formation, a relatively high level of purine nucleotide is required. It will be shown that the association DNA-enzyme complex differs from the initiation DNA-enzyme complex. The process of initiation is rate-limiting at low nucleoside triphosphate concentrations and purine nucleotides in relatively high concentrations overcome this limitation. In step 3, there is no differential effect of purine over pyrimidine nucleotides. The effect of purine nucleotides on initiation correlates with the observations of Maitra and Hurwitz[1] that the purine nucleoside triphosphates are the predominant 5′-terminal nucleotides found in RNA synthesized *in vitro*.

Materials and Methods.—Nucleoside triphosphates (NTP) unlabeled were obtained from the Sigma Chemical Company (type 1). P^{32}-NTP's were purchased from the International Chemical and Nuclear Corp., and H^3-CTP was obtained from Schwarz BioResearch, Inc. A specific activity of 1,500–4,000 cpm/mμmole was employed.

E. coli polymerase was prepared according to the procedure of Hurwitz[2] and had a specific activity of 3,900–5,250 units/mg/hr. Two types of assays were employed to measure the synthesis of RNA. The first was the standard procedure as noted below Table 1. The second involved a membrane filter technique. Reaction mixtures were preincubated under the conditions noted in Table 2. The reactions were then temporarily terminated by filtration of the mixtures on 1.2-μ Millipore membranes. The DNA-enzyme complexes were retained while free enzyme or DNA passed through the filters.[3] The complexes were washed with 0.05 *M* potassium maleate buffer pH 7.5, 0.001 *M* mercaptoethanol at 0°, and the membranes were then introduced into reaction mixtures containing nucleoside triphosphates, one of which was isotopically labeled, plus metals, buffer, and mercapto-

TABLE 1

INHIBITION OF ASSOCIATION BY $(NH_4)_2SO_4$ AND FORMATION OF A STABLE COMPLEX BY INITIATION WITH PURINE NUCLEOTIDES

	Preincubation mixture (time 2′)	Additions during incubation (time 10′)	Incorporation (mμmoles)	Control (%)
1	⋮	Enz, DNA, AGUC	1.44	100
2	DNA, AGUC, $(NH_4)_2SO_4$ →	Enz.	0.005	1
3	Enz, DNA, AGUC →	$(NH_4)_2SO_4$	0.41	29
4	Enz, DNA, AG →	$(NH_4)_2SO_4$, UC	0.33	23
5	Enz, DNA, UC →	$(NH_4)_2SO_4$, AG	0.09	6
6		Enz, DNA, AG	0.007	1

The reaction mixtures contained in 0.5 ml 50 mM potassium maleate (pH 7.5), 2 mM $MnCl_2$, 8 mM $MgCl_2$, 5.4 mM mercaptoethanol, 0.4 mM each of three unlabeled NTP's, 0.1 mM H^3-CTP or P^{32}-GTP (1600 cpm/mμmole and 1720 cpm/mμmole, respectively), 25 μg calf thymus DNA, 0.014 mg enzyme (4,650 units/mg/hr at 38°C), and, where indicated, $(NH_4)_2SO_4$ 0.4 *M*. Temperature was 28°C. The sequence of additions was as indicated in each experiment; where isotope was present in the preincubation, a correction for this was made by measuring the amount of incorporation during this period in control tubes and the mμmoles reported represent the incorporation of isotope during the ensuing 10′ incubation period. Reactions were terminated by precipitation in 5% TCA at 0°C. Precipitates were filtered and washed with 5% TCA on Millipore membranes and counted in a scintillation system. A = ATP, G = GTP, C = CTP, U = UTP.

Incorporation in mμmoles as well as units of enzyme activity is based on the incorporation of the labeled nucleotide.

ethanol. After incubation, the reactions were stopped by washing the membranes with cold 5 per cent TCA. Experiments demonstrated that there was no loss of RNA product from the membranes in incubations of short duration.

Data obtained by the membrane technique were averaged from duplicate determinations; data obtained by the routine assay were either from duplicate determinations or confirmed by repeat experiments.

Results.—Association of the RNA polymerase with DNA: The association complex (DNA-enzyme) can be differentiated from the complex formed during initiation and polymerization by the effects of high ionic strength. The equilibrium between DNA plus free enzyme and the complex lies far toward the complex. However, when 0.4 *M* $(NH_4)_2SO_4$ was added to the reaction mixtures prior to the addition

TABLE 2

THE EFFECT OF PURINE NUCLEOTIDES ON THE FORMATION OF INITIATION COMPLEXES ISOLATED ON NITROCELLULOSE MEMBRANES

Expt. no.	Preincubation (2′) unlabeled NTP's (0.2 mM)	Incubation (8′) Incorporation (mμmoles)	Incubation (8′) Incorporation (Δ mμmoles)	Increase, %
1	None	0.028	—	—
2	AGUC	0.152	0.124	443
3	A	0.039	0.011	39
4	G	0.048	0.020	71
5	U	0.032	0.004	14
6	C	0.034	0.006	21
7	AG	0.102	0.074	264
8	AU	0.059	0.031	111
9	AC	0.044	0.016	57
10	GU	0.06	0.032	114
11	GC	0.046	0.018	64
12	CU	0.04	0.012	42
13	AGU	0.104	0.076	271
14	ACU	0.096	0.068	243
15	GCU	0.067	0.039	139

Preincubation: mixtures containing unlabeled NTP's, as indicated in column 2, 50 mM potassium maleate (pH 7.5), 2 mM $MnCl_2$, 8 mM $MgCl_2$, 5.4 mM mercaptoethanol, 50 μg calf thymus DNA, and 0.038 mg enzyme (5250 units/mg) in a volume of 1.0 ml were incubated for 2 min at 28°C. Then 0.8 ml of the reaction mixture was filtered and washed on a nitrocellulose membrane as described in *Materials and Methods*.

Incubation: The membrane with the adsorbed complex was added to 0.8 ml of reaction mixture containing 0.01 mM each of ATP, GTP, UTP, and H^3-CTP (1480 cpm/mμmoles) and buffer, metals, and mercaptoethanol as in the preincubation. Incubation was for 8 min at 28°C.

of enzyme, incorporation of radioactive nucleotide was almost completely inhibited (compare expts. 1 and 2, Table 1). Inhibition similar to experiment 2 was noted with the enzyme present during the preincubation when $(NH_4)_2SO_4$ was added prior to either DNA or nucleotides, and incorporation was then determined during the ensuing 10 min incubation. Direct evidence for the dissociation of the DNA-enzyme complex by $(NH_4)_2SO_4$ was obtained using the nitrocellulose membrane technique. The membrane adsorbs the association or initiation complex but does not retain free DNA or enzyme. When the association complex of C^{14}-DNA and enzyme was added to solutions of increasing $(NH_4)_2SO_4$ concentration and the mixture filtered, it was observed that a concentration of 0.266 *M* $(NH_4)_2SO_4$ prevented retention of C^{14}-DNA. This indicated that complete dissociation of the complex had occurred. Since KCl (0.266 *M*) also dissociated the DNA-enzyme complex, it seems reasonable to conclude that high ionic strength inhibits the reaction primarily by inhibiting the process of association. Similar conclusions have been drawn by other workers.[3, 4]

Initiation and polymerization: Evidence indicates that initiation leads to a stabilized form of the DNA-enzyme complex which is not dissociated by $(NH_4)_2SO_4$. This is indicated as a DNA-enzyme-nucleotide complex. It is seen in experiment 3 of Table 1 that if all the components for synthesis were present for 1 min prior to addition of $(NH_4)_2SO_4$, 0.41 mμmole of the labeled nucleotide was incorporated during the ensuing 10 min incubation as compared to 0.005 mμmole (expt. 2) when association was blocked with $(NH_4)_2SO_4$. Thus, initiation permitted polymerization equivalent to 29 per cent of the synthesis which occurred in the absence of $(NH_4)_2SO_4$ (expt. 1, Table 1). (One reason why this is only 29% is because $(NH_4)_2SO_4$ also has an inhibitory effect upon the rate of polymerization, which is related to the nucleotide concentration.) It is also evident that purine nucleotides alone added prior to $(NH_4)_2SO_4$, but not pyrimidine nucleotides, allowed almost maximum synthesis (compare expts. 4 and 5, with expt. 3, Table 1). No significant synthesis occurred with ATP plus GTP in the absence of the pyrimidine nucleotides (expt. 6). Since ATP and GTP were able to prevent the dissociation of a DNA-enzyme complex by $(NH_4)_2SO_4$, it seems most reasonable to postulate that a DNA-enzyme-purine nucleotide complex was formed.

Further evidence which indicates that the effect of purines is on initiation was obtained by the membrane technique described in *Materials and Methods*. For the data in Table 2, reaction mixtures were preincubated with unlabeled nucleotides as indicated in the second column, and then filtered and washed on Millipore membranes. Each membrane was then added to a reaction mixture containing 0.01 mM of each of the four nucleoside triphosphates (one of which was labeled), but no additional enzyme or DNA. When the concentration of all four nucleoside triphosphates in the preincubation mixture was 0.2 mM, there was a 443 per cent increase in synthesis of RNA (expt. 2) relative to synthesis on membranes which contained enzyme and DNA that were preincubated in the absence of nucleoside triphosphates (expt. 1). Preincubation which included ATP plus GTP resulted in a 264 per cent increase (expt. 7), but preincubations with UTP plus CTP gave only a 42 per cent increase (expt. 12). Effects of preincubation with ATP or GTP alone (expt. 3 and 4) were less than when they were together (expt. 7), which suggested that the 264 per cent increase was not due to the binding of single nucleotides but due to some interaction. Since the effect of purines was observed only when con-

ditions were varied before addition of the radioactive isotope, this effect must be due to initial events in the synthesis of polymer.

Direct evidence that addition of purine nucleotides stabilized the DNA-enzyme complex was obtained by examining the effects of $(NH_4)_2SO_4$, in the presence and absence of nucleotides, on retention of these complexes by nitrocellulose membranes. In the absence of $(NH_4)_2SO_4$ or nucleotides, 1.60 μg of T4 C^{14}-DNA was retained by these membranes as a DNA-enzyme complex. When the preformed complex was incubated in 0.45 *M* $(NH_4)_2SO_4$ and filtered, less than 0.16 μg of C^{14}-DNA was adsorbed to the membrane. If the complex was first preincubated with the four nucleoside triphosphates and then incubated in 0.45 *M* $(NH_4)_2SO_4$ and filtered, 1.60 μg of C^{14}-DNA was retained by the membrane. A preincubation with ATP plus GTP resulted in adsorption of 1.07 μg of C^{14}-DNA or of 66 per cent of the complex. In contrast, when the preincubation was in the presence of UTP plus CTP, only 0.21 μg of C^{14}-DNA was retained; that is, only 13 per cent of the complex remained stable to $(NH_4)_2SO_4$.

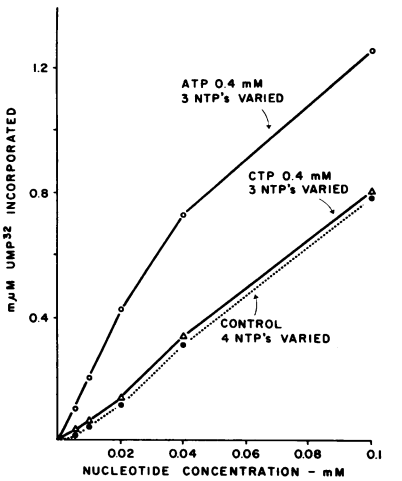

FIG. 1.—Kinetic demonstration of initiation by ATP. Reacion mixtures contained: buffer, metals, DNA, and mercaptoethanol as in Table 1 and 0.014 mg enzyme (3900 units/mg). The NTP's including P^{32}-UTP were varied and incubations were for 4 min at 38°C. Except where indicated, the concentration of each of the four nucleotides is the same and is plotted on the abscissa.

Thus, by both an ammonium sulfate and a membrane technique as well as a combination of these two techniques, evidence has been provided for the existence of a DNA-enzyme-purine complex. Experiments are in progress to demonstrate directly the presence of purine nucleotides in this complex.

Initiation could also be demonstrated kinetically (Fig. 1). When the concentration of each of the four nucleotides was the same, and this concentration was varied, a sigmoid curve was obtained (Fig. 1, *control*). However, if the concentration of ATP was fixed at 0.4 mM and the remaining three nucleotides were varied, incorporation was stimulated and normal Michaelis-Menten kinetics were obtained. A similar experiment with CTP at 0.4 mM showed no stimulation. In these experiments calf thymus DNA was used as template, and the effect was observed with single-stranded as well as double-stranded DNA. When a single nucleoside triphosphate was 0.4 mM and the other three 0.01 mM, the per cent stimulation relative to the rate of incorporation with the four nucleoside triphosphates at 0.01 mM was as follows: ATP, 128 per cent; GTP, 95 per cent; UTP, 44 per cent; and CTP, 12 per cent. In addition, using this technique, it has been shown that the stimulation noted with the respective purines varies with the DNA template. A comparison of calf thymus DNA with *Micrococcus lysodeikticus* DNA is shown in Table 3. Maitra and Hurwitz have shown that the first nucleotide incorporated into RNA with calf thymus DNA as template is primarily GTP or ATP, while with *M. lysodeikticus* DNA it is primarily GTP.[1] It is apparent that both purine nucleoside triphosphates stimulate with calf thymus DNA, but primarily GTP stimulates with *M. lysodeikticus* DNA.

TABLE 3

STIMULATORY EFFECTS OF PURINE NUCLEOTIDES ON RNA SYNTHESIS DIRECTED BY CALF THYMUS AND *M. lysodeikticus* DNA COMPARED WITH THE 5'-TERMINAL NUCLEOTIDES OF RNA SYNTHESIZED *in vitro*

Nucleoside Triphosphate Conc. (0.2 mM)	(0.01 mM)	Calf Thymus DNA: Stimulation over control, %	Calf Thymus DNA: γP^{32} NTP terminus, %*	*M. lysodeikticus* DNA: Stimulation over control, %	*M. lysodeikticus* DNA: γP^{32} NTP terminus, %*
—	AGUC	Control	—	Control	—
A	GUC	154	29.8	66	11.7
G	AUC	81	50.4	257	81.2
C	AGU	13	7.0	16	3.9

Reaction mixtures contained: 0.019 mg of enzyme (5250 units/mg), 25 μg of calf thymus or *M. lysodeikticus* DNA, and nucleoside triphosphates as indicated. Contents were otherwise the same as in Table 1. P^{32}-UTP was 1290 cpm/mμmole. Incubations were for 10 min at 28°C.

* These values refer to the nucleotide indicated in column 1 of this table and represent the per cent of the total 5'-terminal ribonucleotides. These data were taken from Maitra and Hurwitz (ref 1).

Evidence has been obtained that a lag phase in RNA synthesis occurs under certain conditions and that this lag may be due to a rate-limiting step in initiation. With calf thymus DNA, when all nucleoside triphosphates were present at 0.01 mM, the lag occurred prior to linear synthesis (Fig. 2). This lag phase decreased when the concentration of the four nucleoside triphosphates was increased and was absent at a concentration of 0.4 mM. It was also eliminated when the ATP concentration was 0.2 mM and the remaining nucleoside triphosphates were 0.01 mM (Fig. 3). CTP (0.2 mM) did not alter the lag phase. In addition to the effect upon the lag phase it should be noted that there was an increase in the rate of

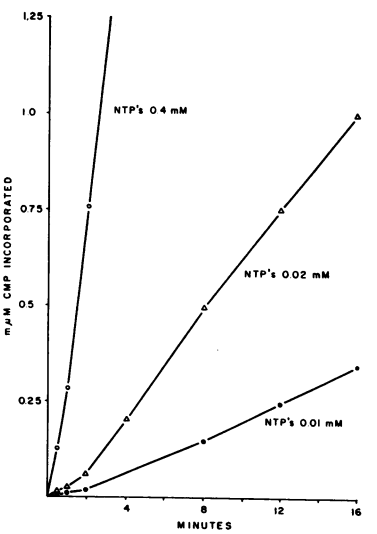

FIG. 2.—Effect of nucleotide concentration on the lag due to rate limitation by initiation. Reaction mixtures contained buffer, metals, DNA, and mercaptoethanol as in Table 1, 0.019 mg enzyme (5250 units/mg) and NTP's with H^3-CTP as indicated in 0.5 ml. Incubations were at 28°C. ●—● four NTP's, 0.01 mM; Δ—Δ four NTP's, 0.02 mM; O—O four NTP'S, 0.4 mM. At 4 min, with four NTP's at 0.4 mM, 1.41 mμmoles of CMP were incorporated.

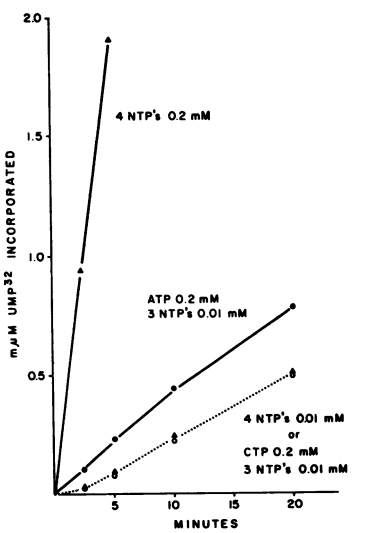

FIG. 3.—Elimination of the lag in the rate of RNA synthesis by an elevated ATP concentration. Reaction mixtures were similar to those in Fig. 2 except for concentrations of NTP's with P^{32}-UTP as noted in the figure. Incubations were at 28°C. Δ—Δ four NTP's, 0.2 mM; ●—● ATP, 0.2 mM; and three NTP's, 0.01 mM; ▲—▲ CTP, 0.2 mM; and three NTP's, 0.01 mM; O—O four NTP's, 0.01 mM.

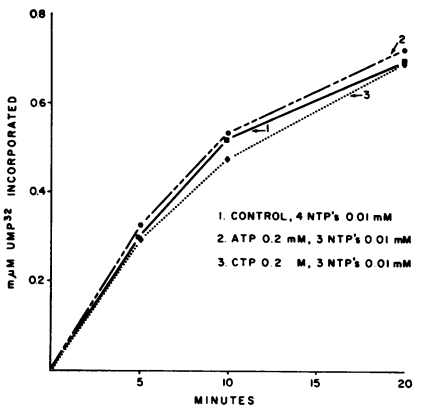

FIG. 4.—The effect of ATP and CTP on the polymerization which occurs on nitrocellulose membranes. Preincubation: mixtures containing each of the four unlabeled NTP's, 0.2 mM, 50 mM potassium maleate (pH 7.5), 2 mM $MnCl_2$, 8 mM $MgCl_2$, 5.4 mM mercaptoethanol, 50 μg calf thymus DNA, and 0.038 mg enzyme (5250 units/mg) in a volume of 1.0 ml were incubated for 2 min at 28°C. Then 0.8 ml of each reaction mixture was filtered and washed on a nitrocellulose membrane as described in *Materials and Methods*.

Incubation: The membrane with the adsorbed complex was added to 0.8 ml of reaction mixture containing NTP's as indicated in the figure including P^{32}-UTP (2020 cpm/mμmole) and buffer, metals, and mercaptoethanol as in the preincubation. The incubation was at 28°C.

synthesis with increasing substrate concentration which was greater than the two-fold rise predicted when the nucleotide concentration was varied from 0.01 mM to 0.02 mM (Fig. 2).

A number of possible explanations for the purine effect other than its relationship to initiation have been ruled out. Contamination of nucleoside triphosphates was excluded by the observation that a similar order of stimulation of incorporation was noted with nucleoside triphosphates purified by Dowex 1-Cl column chromatography. Also, the purified ATP and GTP stabilized the DNA-enzyme complex against dissociation by $(NH_4)_2SO_4$. The stimulatory effect of ATP was seen when UTP, CTP, or GTP was isotopically labeled. Finally, the nearest neighbor patterns obtained with P^{32}-UTP at high and low concentrations of ATP were not significantly different.

There was no specific purine nucleotide stimulation of the process of polymerization *per se*. To demonstrate this (Fig. 4), uniform initiation was achieved by a 2-min preincubation in unlabeled nucleoside triphosphates at 0.2 mM; the reaction mixtures were then filtered on Millipore membranes to remove free enzyme. These membranes were then introduced into reaction mixtures containing either 0.2 mM ATP or CTP and the remaining nucleoside triphosphates including the isotopic nucleotide, at 0.01 mM. A control with the four nucleoside triphosphates at 0.01 mM was included. No stimulation by ATP was noted, and it can be concluded that the stimulation of RNA synthesis noted with ATP (Figs. 1 and 3) is not due to an effect upon polymerization. Further evidence that purines do not have an allosteric effect on the polymerization process was obtained with the homopolymer dI:dC, kindly provided by Dr. F. J. Bollum. When CTP was the only nucleotide in the reaction mixture and CMP incorporation was examined, ATP had no stimulatory effect. In this instance, when a homopolymer was used instead of DNA, both initiation and polymerization occurred with CTP. This, plus the data with different DNA's, means that the information for the 5'-terminal nucleotide must reside primarily in the DNA template.

Discussion.—Association, initiation, and polymerization are three separate steps in the process of RNA synthesis. Association can be inhibited by high ionic strength, thus preventing initiation and polymerization. Because of the direct evidence of Maitra and Hurwitz[1] and the indirect evidence presented here that

purine nucleotides effect initiation, it is reasonable to assume that the enzyme has some preferential affinity for pyrimidine nucleotides in both double- and single-stranded DNA. Initiation can be distinguished from polymerization by the stimulatory effects of a relatively high concentration of purine nucleoside triphosphates. This purine nucleotide effect can be demonstrated during a preincubation period by the use of $(NH_4)_2SO_4$, by a membrane filter technique or by a combination of these techniques. Which purines are most effective depends upon the DNA employed and correlates with data on the 5′-terminal ribonucleotides incorporated with calf thymus and *M. lysodeikticus* DNA. With calf thymus DNA a lag in synthesis can be overcome by high levels of ATP, but not by CTP, which suggests that initiation can be a rate-limiting step.

With calf thymus DNA, the effect of both of the purine nucleotides, using the membrane technique, is far more than additive. This suggests that the effect observed with both ATP and GTP may be due to the formation of the first phosphodiester bond. In order to form the first phosphodiester bond it is necessary to have two nucleoside triphosphates bound to two separate sites on the enzyme. The 5′-terminal nucleotide remains as a triphosphate,[1] and the second nucleotide splits out pyrophosphate in the formation of the phosphodiester bond. One reason for the high level of purine nucleotide required to initiate synthesis may be the requirement for two nucleotides to be present on the enzyme at the same time in order to form this phosphodiester bond and the fact that one of these, the terminal, must be a purine nucleotide. In this case, the K_m for both nucleotides could be low, and the requirement for the relatively high molarity of purine nucleotide might be due to a concentration effect in a bimolecular reaction with the DNA-enzyme complex. An alternative explanation involves a high K_m for the 5′-terminal nucleotide and a low K_m for the second nucleotide. In this model the polymerase must move relative to both the DNA and the RNA product after the first phosphodiester bond is formed. Therefore, the site originally occupied by the 5′-terminal triphosphate would become occupied by the second nucleotide, and the incorporation of the third and subsequent nucleotides would occur only at the second site on the enzyme. In this model, a high K_m for the first site and a low K_m for the second site would account for the present observations. A preliminary examination of some of the parameters of initiation and polymerization has been made. When three of the nucleoside triphosphates were held at a concentration of 0.2 mM and the fourth varied, a K_m for ATP was 0.015 mM, and for CTP was 0.013 mM. However, when the concentrations of all four nucleotides were varied together and ATP was labeled, a K_m of 0.21 mM was obtained. These results are in agreement with data obtained by others.[5-7] In the first case, three nucleoside triphosphates, including a purine, were present in high concentration and the low K_m may be related to polymerization, while in the second case the high K_m may reflect primarily the component of initiation. Some support for this explanation was obtained in preliminary experiments with T4 DNA. When synthesis was initiated on membranes with all nucleotides unlabeled at 0.4 mM, and incorporation was then measured as a function of the varying concentration of all four nucleotides, the lower K_m was obtained. Further kinetic analysis of initiation and polymerization is in progress. This analysis is complicated by the fact that initiation under certain conditions is a continuous process as has been observed in this laboratory and by Maitra and Hurwitz.[1]

Either of the two models which relate the purine nucleotide effects to initiation of synthesis is in keeping with the observation that no specific stimulatory effect of purines could be demonstrated on the process of polymerization.

The level of purine nucleoside triphosphate at which initiation is impaired is below 0.1–0.2 mM. A recent estimate by Neuhard and Munch-Petersen of ATP and GTP concentrations in exponentially growing cells is 3.07 ± 0.98 and 1.18 ± 0.41 μmoles per gm of dry weight.[8] It would be of interest to know whether there are any physiological conditions in which messenger RNA synthesis is inhibited because initiation is impaired.

The techniques described in this paper should permit an examination of initiating regions in native DNA's from different species.

Summary.—The action of RNA polymerase has been separated into three steps, association, initiation, and polymerization.

* Supported by a grant from the National Institutes of Health, GM 06075.

† American Cancer Society fellow.

‡ National Institutes of Health Research Career Investigator.

[1] Maitra, U., and J. Hurwitz, these PROCEEDINGS, **54**, 815 (1965).

[2] Hurwitz, J., personal communication.

[3] Jones, W. O., and P. Berg, personal communication.

[4] Chambon, P., M. Ramuz, and J. Soly, *Biochim. Biophys. Res. Commun.*, **21**, 156 (1965).

[5] Chamberlin, M., and P. Berg, these PROCEEDINGS, **48**, 81 (1962).

[6] Furth, J. J., J. Hurwitz, and M. Anders, *J. Biol. Chem.*, **237**, 2611 (1962).

[7] Fox, F. E., and S. B. Weiss, *J. Biol. Chem.*, **239**, 175 (1964).

[8] Neuhard, J., and A. Munch-Petersen, *Biochim. Biophys. Acta*, **114**, 61 (1966).