Studies with the Ribonucleic Acid Polymerase. II. Kinetic Aspects of Initiation and Polymerization*

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ABSTRACT: Kinetic studies of ribonucleic acid synthesis with the deoxyribonucleic acid dependent ribonucleic acid polymerase of *Escherichia coli* are presented in this paper. A nonlinear double-reciprocal plot of nucleotide incorporation as a function of the concentration of the four nucleoside triphosphates was observed. The plot became linear and the amount of incorporation was increased, particularly at low nucleotide concentrations, if a single nucleotide was fixed at a high concentration and if this nucleotide was one of those present in a significant percentage of the 5′-terminal nucleoside triphosphate positions. This effect on the double-reciprocal plot was demonstrated with adenosine triphosphate and calf thymus deoxyribonucleic acid, guanosine triphosphate and *Micrococcus lysodeikticus* deoxyribonucleic acid, and adenosine triphosphate and dAT copolymer. Linear plots were also obtained if the deoxyribonucleic acid–enzyme complex was preincubated with unlabeled nucleotides to initiate ribonucleic acid synthesis and the complexes were then filtered on nitrocellulose membranes and incubated with varying concentrations of the four nucleotides. This was observed with T4, calf thymus, and *M. lysodeikticus* deoxyribonucleic acid. Apparent K_m values were obtained when three nucleotides were fixed at 0.4 mM and the fourth varied. With *M. lysodeikticus* deoxyribonucleic acid, where more than 85% of the 5′-terminal nucleotide is guanosine triphosphate, an apparent K_m for guanosine triphosphate was 0.15 mM while the apparent K_m for adenosine triphosphate, cytidine triphosphate, or uridine triphosphate was approximately 0.015 mM. With prior initiation with the four unlabeled nucleotides and *M. lysodeikticus* deoxyribonucleic acid, the apparent K_m for guanosine triphosphate decreased to 0.027 mM. A tentative model has been proposed. Initiation of ribonucleic acid synthesis involves the formation of the first phosphodiester bond. The apparent K_m for the 5′-terminal nucleotide is approximately 0.15 mM. Polymerization involves migration of the enzyme on the deoxyribonucleic acid and the addition of subsequent nucleotides. An apparent K_m for nucleotides for polymerization is approximately 0.015 mM or one-tenth the concentration for initiation.

Association, initiation, and polymerization are three steps in the synthesis of RNA by the DNA-dependent RNA polymerase. In preliminary experiments, these steps have been separated and analyzed *in vitro* by kinetic studies (Anthony *et al.*, 1966). *Association* defines the formation of the complex of RNA polymerase and DNA. This DNA–enzyme complex can be dissociated with relatively high ionic strength such as 0.4 M $(NH_4)_2SO_4$.

Initiation is defined in this paper as the addition of both the 5′-terminal and the subterminal nucleoside triphosphates to the DNA–enzyme complex and the formation of the first phosphodiester bond. Other workers have studied initiation by examining the 5′-terminal nucleoside triphosphate and have shown that it is primarily a purine nucleotide (Maitra and Hurwitz, 1965). In this paper, when a single nucleotide is described as involved in initiation, it is considered to be at the 5′-terminal position. Previous studies in this laboratory established that ATP plus GTP would stabilize the calf thymus or T4 DNA–enzyme complex to high ionic strength whereas UTP plus CTP were relatively ineffective (Anthony *et al.*, 1966). It was also shown that ATP at 0.2 M stimulated RNA synthesis (directed by calf thymus DNA) when the other three nucleotides were at 0.01 mM, while CTP at 0.2 mM in a comparable experiment had no significant stimulatory effect. Furthermore this differential stimulatory effect of ATP compared to CTP could be eliminated by the preincubation of the DNA–enzyme complex with the four unlabeled nucleoside triphosphates at 0.2 mM and filtration of the nucleic acid–enzyme complexes on membranes prior to measuring RNA synthesis with elevated levels of either ATP or CTP and the other nucleotides at 0.01 mM. These kinetic results suggested that purine nucleotides had a differential effect compared to pyrimidine nucleotides on initiation.

Polymerization is defined as synthesis of RNA following initiation. For polymerization, lower levels of nucleoside triphosphates are sufficient.

In this communication, an analysis of the complex kinetics observed when the concentrations of nucleoside triphosphates are varied is presented. First, experiments are shown which demonstrate that the nonlinear double-

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reciprocal plot of velocity *vs.* nucleotide concentration is related to initiation and, if adequate initiation occurs, the plot becomes linear. Secondly, an attempt is made to evaluate what will be called apparent K_m values for initiation and for polymerization.

Experimental Procedure

Enzyme. RNA polymerase was purified from *Escherichia coli* W (Maitra and Hurwitz, 1967). The enzyme used had a specific activity with calf thymus DNA in the range of 4000–6000 units where a unit was defined as the amount of enzyme required for the incorporation of 1 mμmole of labeled nucleoside monophosphate in 60 min at 37°. Protein concentration was determined by two methods (Lowry *et al.*, 1951; Warburg and Christian, 1942). The ratio of enzyme protein concentration determined by the Lowry procedure (with bovine serum albumin as a standard) to enzyme protein concentration determined by optical density was 1.4. All specific activities are reported for proteins determined by the Lowry method.

DNA. Calf thymus DNA was prepared (Hurst, 1958). Single-stranded calf thymus DNA was prepared by heat denaturation. The DNA was heated 10 min at 100° and cooled to 0° immediately. DNA of bacteriophage T4 and T7 was prepared (Mandell and Hershey, 1960). *Micrococcus lysodeikticus* DNA was extracted (Marmur, 1961) by a modified procedure.[1] It was also purchased from the Miles Chemical Co. and after solubilization was filtered though Millipore membranes.

Nucleotides. Unlabeled nucleoside triphosphates, isolated from equine muscle, were purchased from the Sigma Chemical Corp. [^{3}H]CTP and [^{3}H]GTP were obtained from Schwarz BioResearch Inc. [^{32}P]UTP and [^{32}P]GTP were purchased from International Chemical and Nuclear Corp. The isotopic compounds were purified by paper chromatography with a solvent system of isobutyric acid–1 M NH_4OH (3:1).

Assays. Additions to the reaction mixtures are indicated in the legends. At the end of a reaction a 0.3-ml aliquot was added to 5.0 ml of cold 5% trichloroacetic acid to stop the reaction and this was filtered on a Millipore filter (RA 1.2 μ). The membrane was then washed six times with 5.0-ml aliquots of cold 5% trichloroacetic acid. Counting of both ^{32}P and tritium isotopes was done in a Packard Tri-Carb liquid-scintillation counter.

The technique of filtration of a DNA–enzyme complex on a membrane and the use of this complex to measure activity is an adaptation of the original observation of Jones and Berg (1966) that the DNA–enzyme complex was retained by Millipore membranes. The procedure has been described (Anthony *et al.*, 1966) and further details are in preparation.

Evidence That Nucleotide Stimulation Is Not an Artifact. A number of different explanations for the stimulation of RNA synthesis by elevated nucleotide levels have been ruled out. Contamination of the nucleoside triphosphates was considered. Each of the four nucleoside triphosphates was repurified by column chromatography on Dowex 1, under conditions which separated them (Hurlbert, 1957). When these nucleotides were tested at elevated concentrations for a stimulatory effect on RNA synthesis, the results were similar to those obtained when commercially available nucleotides were used.

The synthesis of poly A and other polymers was also considered as an explanation for the stimulatory effects of elevated nucleotide levels. The following experiments make this unlikely. First, a similar stimulatory effect was noted due to ATP when UTP, CTP, or GTP was isotopically labeled. Second, a nearest-neighbor analysis (Hurwitz *et al.*, 1962) with [α-^{32}P]UTP showed that the pattern of neighbors obtained with calf thymus DNA was not altered significantly by high levels of ATP. With all nucleotides at 0.01 mM the distribution of ^{32}P was AMP 30%, GMP 14%, UMP 34%, and CMP 22%. With ATP at 0.2 mM, which produced a stimulation of 115%, the pattern was AMP 31%, GMP 18%, UMP 30%, and CMP 21%. Third, the stimulation by ATP was greater than by GTP with calf thymus and T4 DNA while this was reversed with T7 DNA and especially with *M. lysodeikticus* DNA.

Polynucleotide phosphorylase contamination was also considered. If this were responsible for any of the incorporation, then isotopically labeled nucleoside diphosphate would be the substrate. When isotopically labeled CTP was used to measure RNA synthesis, addition of unlabeled CDP did not depress incorporation of the isotope, thus ruling out this possibility.

Results

Nucleoside Triphosphate Specificity for the Stimulation of RNA Synthesis. Initiation of RNA synthesis with different DNA templates has been studied in other laboratories by the direct observation of the nature of the 5′-terminal nucleoside triphosphate. Purine nucleoside triphosphates were noted to predominate and the amount of ATP *vs.* GTP varied with the source of the DNA (Maitra and Hurwitz, 1965). In the preliminary studies on the kinetics of initiation, it was observed that varying degrees of stimulation of RNA synthesis were observed when the concentration of one nucleoside triphosphate was fixed at 0.2–0.4 mM while the other three were 0.01 mM. A more complete analysis of this stimulatory effect is presented in Table I. Stimulation by ATP was greater than by GTP with calf thymus and T4 DNA while with T7 and *M. lysodeikticus* DNA, stimulation with GTP was greater than with ATP. With all DNAs both purine nucleotides at the higher concentration produced far more incorporation than both pyrimidine nucleotides at the higher concentration. For these experiments, the DNAs were present in concentrations which would saturate reaction mixtures that contained all four nucleotides at 0.4 mM. Thus from the data shown in Table I, it is apparent that the source of the DNA is related to the type of nucleotide stimulation.

[1] L. Grossman, personal communication.

TABLE I: Stimulation of Nucleotide Incorporation by Different Nucleoside Triphosphates in High Concentration with DNA from Different Sources.[a]

Concn of Nucleoside Triphosphate		% Stimulation of Incorporation Above Control Values[b]			
0.2 mM[c]	0.01 mM	Calf thymus DNA	T4 DNA	T7 DNA	*M. lysodeikticus* DNA
	AGUC	Control (0)	Control (0)	Control (0)	Control (0)
A	GUC	128	100	114	20
G	AUC	95	56	146	460
U	AGC	44	3	63	0
C	AGU	12	10	34	88
AG	UC	424	260	276	870
UC	AG	113	17	96	68

[a] This table is a composite of experimental results; data for each DNA were obtained in a single series of experiments. All incubations, 0.5 ml in volume, contained buffer, metals, and mercaptoethanol as indicated in the legend to Figure 1 and nucleotides at the concentration indicated in this table including a labeled nucleoside triphosphate. Experiments with calf thymus DNA contained 19 μg of DNA and 30 μg of enzyme; experiments with T4 phage DNA contained 35 μg of DNA and 14 μg of enzyme; experiments with T7 phage DNA contained 11 μg of DNA and 7.5 μg of enzyme; and experiments with *M. lysodeikticus* DNA contained 28 μg of DNA and 7.5 μg of enzyme. Different enzyme preparations with different specific activities were used with different DNA preparations, and in each case a saturating level of DNA was used. Incubations were for 5 min at 28°. [b] 100% stimulation means a value for incorporation twice the control value. Control values for different DNAs were as follows: calf thymus 0.106 mμmole; T4 0.09 mμmole; T7 0.108 mμmole; and *M. lysodeikticus* 0.104 mμmole. [c] Elevated concentration of 0.4 mM was used in experiments with *M. lysodeikticus* DNA.

The Nonlinear Double-Reciprocal Plot of RNA Synthesis as a Function of Nucleotide Concentration and Its Relationship to Initiation. In the initial studies with calf thymus DNA, a sigmoid curve was obtained when RNA synthesis was plotted against the concentration of the four nucleotides (Anthony *et al.*, 1966). A double-reciprocal plot of RNA synthesis (in this case millimicromoles of [^{32}P]UMP incorporated in the initial 4 min of incubation) *vs.* nucleotide concentration is shown in Figure 1 (control). This plot is nonlinear. One factor contributing to the nonlinearity was an initial lag period in incorporation when the nucleotide concentration was lower than 0.1 mM. At 0.01 mM this lag was slightly less than 1 min. Following such a lag period, the rate was constant for at least 10 min. At nucleotide concentrations higher than 0.01 mM, shorter lag periods followed by linear rates were observed. If these linear rates, observed with the four nucleotides at 0.01, 0.02, and 0.10 mM, were plotted as a function of substrate concentration in the double-reciprocal form, the plot was still nonlinear. This indicated that the lag was not the only cause of the nonlinearity. Control experiments were also done which demonstrated that the nonlinearity was not due to a variable concentration of free divalent metal related to the variation of nucleotide concentration.

As indicated in Figure 1, when the concentration of ATP was held constant at 0.4 mM and the concentration of the other three nucleotides varied, stimulation of incorporation was observed and furthermore the double-reciprocal plot became linear. Higher concentrations of ATP inhibited RNA synthesis, apparently competitively with the other nucleotides, and the double-reciprocal plots approached linearity. The stimulatory effect and the elimination of nonlinearity by ATP were observed with both single-stranded and double-stranded calf thymus DNA plus either Mg^{2+} (8 mM) or Mn^{2+} (2 mM). With the ATP concentration elevated, and the other three nucleotides at 0.01 mM, there was no initial lag in incorporation.

A similar experiment was done with a concentration of 0.4 mM CTP and variation of the other three nucleotides. Unlike ATP, the CTP did not eliminate the nonlinear response (Figure 2) and there was an initial lag in incorporation. Again higher concentrations of CTP produced inhibition.

Other nucleotides were tested for their stimulatory effect with calf thymus DNA. When ITP was held at a fixed concentration of 0.4 mM and ATP, GTP, CTP, and UTP were varied together from 0.01 to 0.2 mM the reciprocal plot remained nonlinear and only an inhibition of incorporation was noted (60% when the concentration of the four nucleotides was 0.02 mM). dATP at 1.0 mM also inhibited the incorporation and did not alter the nonlinear response; for example, the inhibition was 26% when the four ribonucleotides were at 0.02 mM. At a concentration of 0.4 mM, dATP produced no inhibition when the ribonucleotides were at 0.01 mM. AMP at 0.4 mM produced some inhibition in the rate of incorporation without significant alteration of the nonlinearity. ADP at 0.4 mM did produce a linear reciprocal plot even though incorporation was inhibited. The basis for this has not been investigated.

Thus ATP but not CTP stimulated RNA synthesis

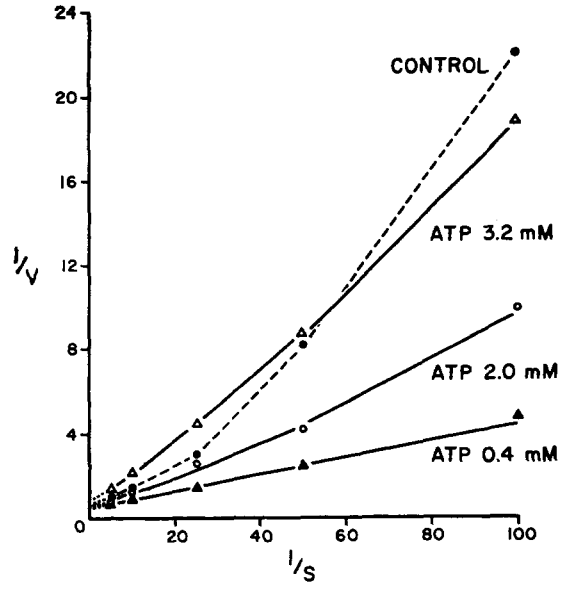

FIGURE 1: The effect of fixed concentrations of ATP on the kinetics of UMP incorporation into RNA measured as a function of increasing concentrations of GTP, UTP, and CTP. Incubation mixtures, 0.5 ml in volume, contained: 50 mM potassium maleate (pH 7.5), 2 mM $MnCl_2$, 8 mM $MgCl_2$, 5.4 mM mercaptoethanol, 20 μg of calf thymus DNA, 14 μg of enzyme, and increasing concentrations of GTP, CTP, and [^{32}P]UTP (4896 cpm/mμmole) from 0.01 to 0.2 mM. ATP was present at the concentrations indicated or in the control at a concentration similar to the other nucleotides. Following a 10-min preincubation at 0°, reactions were started by addition of DNA. Incubations for 4 min at 38° were terminated by addition of a 0.3-ml aliquot to 5 ml of cold 5% trichloroacetic acid on a Millipore filter apparatus. Precipitates were washed with 5% trichloroacetic acid and counted in a scintillation system. The nucleotide concentration is millimolar. Velocity refers to millimicromoles of labeled nucleotide incorporated per reaction mixture during the incubation time.

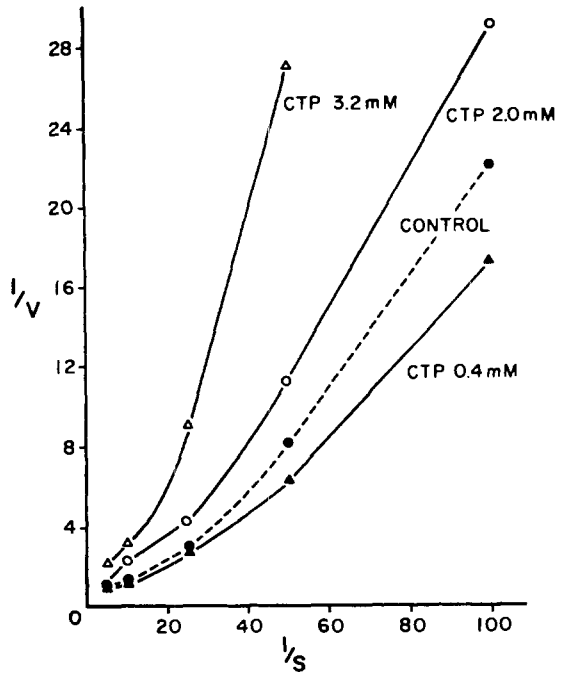

FIGURE 2: The effect of fixed concentrations of CTP on the kinetics of [^{32}P]UMP incorporation into RNA measured as a function of increasing concentrations of ATP, GTP, and UTP. Reaction volume and contents were as reported in the legend to Figure 1, except that the nucleoside triphosphates which were varied in concentration from 0.01 to 0.2 mM were ATP, GTP, and [^{32}P]UTP (2982 cpm/mμmole). CTP was present either at the concentrations designated in the figure or was varied with the other nucleotides (control). Incubations were for 4 min at 38°.

under these conditions and altered the nonlinear double-reciprocal plot. This plus data shown in Table I and information on the 5′-terminal nucleotides of RNA (Maitra and Hurwitz, 1965) suggested that the process of initiation was responsible for the nonlinearity. A series of experiments were then designed to demonstrate that the nonlinear double-reciprocal plot could be made linear, if the process of initiation was not limited by the nucleotide concentration. The first experiments were based on the observation that initiation could be separated from polymerization by a membrane technique (Anthony *et al.*, 1966). It was possible to initiate RNA synthesis with all four unlabeled nucleotides at 0.2 mM, filter the DNA–enzyme–RNA complexes on nitrocellulose membranes, and then introduce these membranes into fresh reaction mixtures which contained all four nucleotides at a concentration which varied from 0.0025 to 0.2 mM. [^{3}H]CTP was present in the second incubation mixtures and incorporation was measured during a 5-min interval. The data are compared with those of a similar experiment in which the concentration of all four nucleotides was varied, but in which there was no prior initiation or filtration on membranes. The results obtained with T4 DNA are shown in Figure 3. It is evident that nonlinear kinetics were obtained without prior initiation, and linear kinetics were observed with prior initiation. Similar data were obtained with calf thymus DNA and with prior initiation there was no lag in nucleotide incorporation when the nucleoside triphosphate concentration was 0.01 mM. These results support the hypothesis that initiation is related to the nonlinear double-reciprocal plot.

The dAT polymer provided another approach to the relationship of initiation to the nonlinear double-reciprocal plot. The 5′-terminal ribonucleotide (95%) observed with dAT as a template was ATP (Maitra and Hurwitz, 1965). The effect of varying ATP and UTP together and separately is shown in Figure 4. Curvilinearity was observed with variation of both nucleotides. With ATP fixed at 0.4 mM and UTP varied, the double-reciprocal plot was linear between 0.004 and 0.05 mM. This linearity would be predicted if initiation was primarily with ATP. An apparent K_m value calculated for UTP was 0.013 mM. However, when UTP was fixed at 0.4 mM and ATP was varied, the plot was not linear, and the rates of incorporation were slower. Thus, the increased rate of incorporation and the linear double-reciprocal plot observed with a fixed concentration of ATP can be interpreted as an effect of ATP on initiation.

Instead of holding one nucleotide at a high concentration and varying the concentration of the other three,

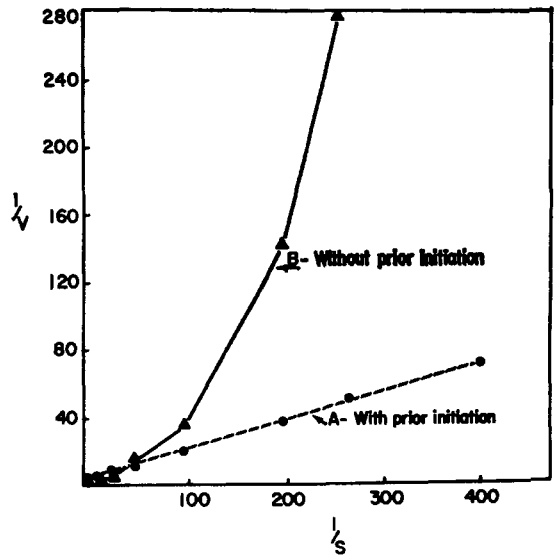

FIGURE 3: The effect of varying the concentration of all four nucleoside triphosphates on the nucleotide incorporation measured with and without prior initiation with T4 DNA. (A) With prior initiation on membranes. Preincubation: Reaction mixtures, 1.0 ml in volume, contained 0.4 mM each of unlabeled ATP, GTP, UTP, and CTP, 38 μg of T4 phage DNA, 32 μg of enzyme, and Mg^{2+}, Mn^{2+}, and Tris buffer as in the legend to Figure 1. After 2 min at 28°, 0.8 ml of each reaction was filtered on a Millipore membrane and the membrane-retained DNA–enzyme complex was washed as in the legend to Figure 1. Incubation: The membranes with adsorbed complexes were added to 0.8-ml reaction mixtures containing ATP, GTP, UTP, and [^{3}H]CTP (3451 cpm/mμmole) in concentrations from 0.0025 to 0.2 mM and buffer, divalent cations, and mercaptoethanol as in the preincubation. Incubations were for 5 min at 28°. (B) Without prior initiation. Reaction mixtures, 0.5 ml in volume, contained buffer, divalent cations, and mercaptoethanol as in the legend to Figure 1, 19 μg of T4 phage DNA, 16 μg of enzyme, and ATP, GTP, UTP, and [^{3}H]CTP in concentrations from 0.0025 to 0.2 mM. Incubations were for 5 min at 28°.

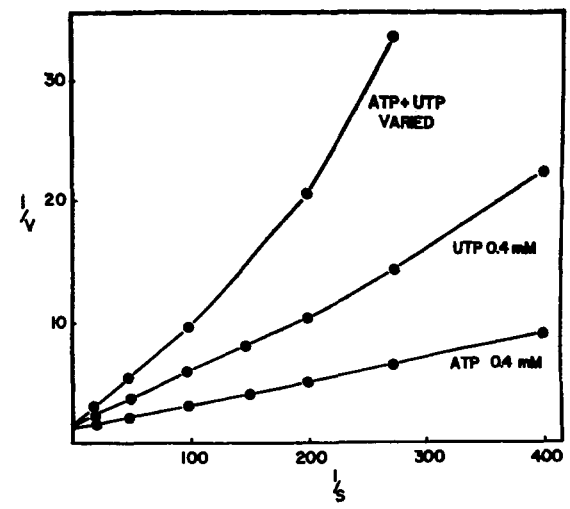

FIGURE 4: The effect of varying ATP and UTP concentrations singly or together on the kinetics of incorporation of nucleotide with the polymer dAT. Incubation mixtures, 0.2 ml in volume, contained buffer, divalent cations, and mercaptoethanol in the concentrations indicated in the legend to Figure 1 with 6.0 μg of dAT and 13 μg of enzyme. UTP and [^{3}H]ATP (3844 cpm/mμmole) were present either in concentrations from 0.0025 to 0.066 mM or 0.4 mM where indicated. Incubations were for 5 min at 28°.

experiments were done with three nucleotides at a high concentration with one varied. If significant initiation occurs with either ATP or GTP, then it might be predicted that the response to the variation of any one nucleotide could reflect polymerization rather than initiation. With calf thymus DNA both ATP and GTP have been shown to be effective in initiation while CTP and UTP were relatively ineffective (Maitra and Hurwitz, 1965). When three nucleotides were kept at 0.2 mM and the concentration of the fourth (either ATP or CTP) was varied, the double-reciprocal plot was linear and the apparent K_m for either ATP or CTP was approximately 0.015 mM (Table II). Slightly lower values have been reported (Hurwitz *et al.*, 1962) for each of the four nucleotides tested in a similar manner. Thus, it is clear that under these conditions the double-reciprocal plots are linear and there is no difference between the apparent K_m for ATP, which initiates, and for CTP, which does not. These values are considered to represent apparent K_m values for polymerization. If adequate initiation occurred under these conditions, one would predict that prior initiation and filtration of the complex on membranes before varying either ATP or CTP would give the same apparent K_m values. Such was the case as shown in Table II. The fact that the apparent K_m values are similar with or without prior initiation also indicates that the microenvironment of the membrane does not affect this property of the enzyme. The most simple interpretation of these experiments with calf thymus DNA is that adequate initiation occurred with GTP when ATP was varied and with GTP plus ATP when CTP was varied and therefore the process of polymerization was examined. This conclusion is supported by similar experiments described below with *M. lysodeikticus* DNA.

Experiments with M. lysodeikticus DNA. GTP is primarily responsible for the initiation process in the presence of *M. lysodeikticus* DNA. Direct measurements of the 5′-terminal nucleoside triphosphates when each of the γ-labeled nucleotides was at 0.16 mM show 85% GTP, approximately 7.5% ATP, 7.5% CTP, and no UTP (Maitra *et al.*, 1967). The data suggest that at higher nucleotide concentrations even more than 85% is GTP. This correlates well with the stimulation of synthesis observed with GTP at 0.4 mM (Table I) which is approximately 81% of the sum of the stimulations observed with each of the four nucleotides. Because initiation with *M. lysodeikticus* DNA is primarily with GTP, this DNA allows a more accurate analysis of the kinetics of initiation and polymerization. Figure 5 shows the double-reciprocal plot obtained when the concentration of all four nucleotides was varied. The insert shows that the plot is linear from 0.15 to 0.40 mM. The rate of incorporation as a function of time is linear when the concentration of the four nucleotides is 0.10 mM and above. Below this level there is a slight lag in incorporation (at 0.01 mM it can be extrapolated to 0.8 min). The

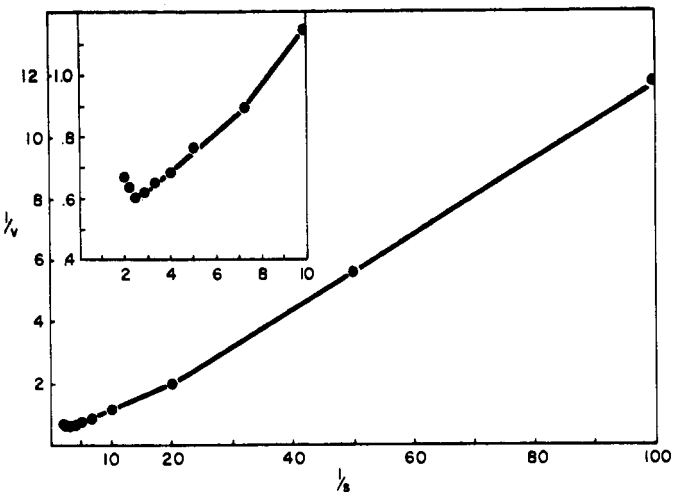

FIGURE 5: The effect of varying the concentration of all four nucleoside triphosphates on the rate of nucleotide incorporation into RNA with *M. lysodeikticus* DNA. Incubation mixtures, 0.5 ml in volume, contained 50 mM Tris-HCl (pH 7.9), 2 mM $MnCl_2$, 8 mM $MgCl_2$, 54 mM mercaptoethanol, 25 μg of *M. lysodeikticus* DNA, 15 μg of enzyme, and increasing concentrations of GTP, ATP, UTP, and [^{3}H]CTP (8000 cpm/mμ mole) from 0.01 to 0.5 mM. Following a 10-min preincubation at 0°, reactions were started by addition of DNA. Incubations for 5 min at 37° were terminated and analyzed as noted in the legend to Figure 1.

TABLE II: Apparent K_m Values for Initiation and Polymerization with Calf Thymus DNA.[a]

Expt	Preincubation: Initiation on Membrane with AGUC at 0.2 mM	Incubation: Nucleoside Triphosphate Concn Fixed 0.2 mM	Incubation: Nucleoside Triphosphate Concn Varied 0.0025–0.2 mM	App K_m
1		GUC	A	0.015
2		AGU	C	0.013
3	+	GUC	A	0.016
4	+	AGU	C	0.022

[a] Reaction mixtures, either preincubated to initiate RNA synthesis and then filtered on membranes or not preincubated (standard reaction assay), were as indicated in the legend to Figure 1 except for the following: the preincubation concentration of nucleoside triphosphates was 0.2 mM, the incubation concentrations of nucleoside triphosphates were as indicated in the table, and 38 μg of calf thymus DNA/ml and 39 μg of enzyme/ml were added. Incubation times for experiments without preincubation were for 8 min; for those with preincubation 5 min; the incubation temperature was 28°.

apparent K_m value calculated from the linear portion of the curve is 0.17 mM.

A Hill plot of these data presented in Figure 5 plus two additional points is shown in Figure 6. An n value of 1.0 was obtained at concentrations of the four nucleotides above 0.10 mM while below this the n value was 1.4 over a range from 0.002 to 0.05 mM. The latter n value is a reflection of both the slight initial lag in incorporation and the altered rate of incorporation after the lag. The importance of the plot lies only in the demonstration that above 0.10 mM the n value is 1.0.

If the nonlinear plot observed in Figure 5 was related to initiation, then prior initiation with filtration of the complexes on membranes, followed by incubation with varying concentrations of the four nucleotides, should give a linear double-reciprocal plot. Such a plot is shown in Figure 7 and the apparent K_m value was 0.030 mM. This response to prior initiation is similar to that seen in Figure 3.

Since GTP is the 5′-terminal nucleotide in such a high percentage of RNA chains initiated, one might predict that if the concentration of GTP plus two other nucleotides was high and the fourth varied, a K_m value representing polymerization would be observed. As shown in Figure 8, apparent K_m values from 0.013 to 0.016 mM for ATP, CTP, and UTP have been found. The double-reciprocal plots were linear with the exception of CTP which produced a diphasic response at the higher concentrations (0.05–0.15 mM). This may be related to the small amount of initiation noted with CTP (Maitra *et al.*, 1967).

In contrast to the results with ATP, CTP, and UTP, it was observed that variation of GTP produced a different linear plot with an apparent K_m of 0.15 mM (Figure 8). At low concentrations of GTP, the rate of RNA synthesis was much slower than at low concentrations of any of the other three nucleotides. The effect of the variation of GTP on the nucleotide incor-

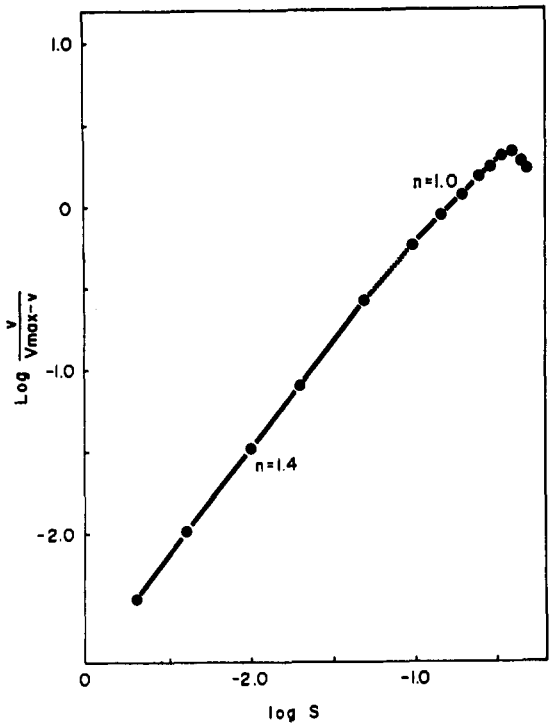

FIGURE 6: A Hill plot of data presented in Figure 5 plus two points obtained at lower concentrations.

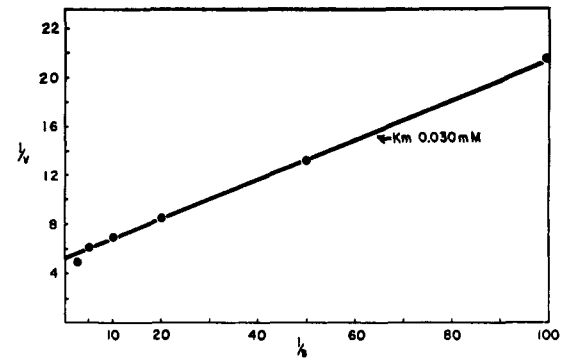

FIGURE 7: The effect of prior initiation on the kinetics of nucleotide incorporation with *M. lysodeikticus* DNA with four nucleoside triphosphates varied. Preinitiation: reaction mixtures, 1.0 ml in volume, containing 0.4 mM each of unlabeled ATP, GTP, UTP, and CTP, 50 μg of *M. lysodeikticus* DNA, 15 μg of enzyme, 50 mM Tris-HCl (pH 7.9), 2 mM $MnCl_2$, 8 mM $MgCl_2$, and 5.4 mM mercaptoethanol were incubated for 2 min at 26°, then 0.8 ml of each reaction mixture was pipetted into 5 ml of 0.4 mM $(NH_4)_2SO_4$ in 0.05 mM Tris-HCl (pH 7.9) with 10^{-3} M mercaptoethanol at 0°. The solution was filtered on a Millipore membrane and the membrane-retained initiation complex was washed with another 5 ml of the same solution and then 20 ml of 0.05 M Tris-HCl (pH 7.9) containing 10^{-3} M mercaptoethanol, at 0°. Incubation: The membrane with the adsorbed complex was added to 0.8 ml of reaction mixture containing ATP, GTP, UTP, and [^{3}H]CTP (1800 cpm/mμmole) in concentrations from 0.01 to 0.4 mM and buffer divalent cations and mercaptoethanol as noted in the preincubation. Incubations were for 5 min at 26°.

poration was considered under these conditions to be primarily on initiation rather than on polymerization. RNA synthesis during the 5-min incubation used for these experiments represented a true rate of nucleotide incorporation since there was no initial lag in the rate of incorporation when either GTP or UTP was at 0.01 mM and the other three nucleotides were at 0.4 mM.

From the direct observations on the 5′-terminal nucleoside triphosphates (Maitra and Hurwitz, 1965), and the kinetic data presented above, it is reasonable to assume that the value of 0.15 mM for GTP represents an apparent K_m for initiation while the low values observed for the other three nucleotides are values for polymerization. These low values are similar to those observed in the presence of calf thymus DNA (Table II).

In the previous experiment with the concentration of GTP varied and that of ATP, CTP, and UTP fixed at 0.4 mM, the apparent K_m for GTP was 0.15 mM. The apparent K_m for each of the other three nucleotides was approximately 0.015 mM. If the high apparent K_m for GTP was related to initiation, then prior initiation by the membrane technique before the determination of the apparent K_m for GTP should decrease this value. The results of such an experiment are shown in Figure 9. Prior initiation decreased the value for GTP to 0.027 mM and the double-reciprocal plot was linear. This is further evidence that the higher apparent K_m for GTP observed in Figure 8 and also in the linear portion of the plot shown in Figure 5 is related to initiation by GTP.

Finally an experiment was done with *M. lysodeikticus* DNA to study the affect of fixing the concentration of one nucleotide at 0.4 mM and varying the other three. The design of this experiment was similar to that shown in Figure 1. The prediction would be a nonlinear double-reciprocal plot for a nucleotide not involved in initiation and a linear plot for an initiating nucleotide. With ATP (which does not initiate significantly with *M. lysodeikticus* DNA) fixed at a concentration of 0.4 mM and the other three nucleotides varied, a nonlinear double-reciprocal plot was observed (Figure 10) which was similar to that seen with variation of all four nucleotides (Figure 5). However, with GTP fixed at 0.4 mM the plot was linear (Figure 10) and the incorporation was stimulated. Since GTP is the predominant 5′ terminal nucleotide, it again is logical to conclude that the linear double-reciprocal plot was due to adequate initiation by the high concentration of GTP and the nonlinear plot with the high ATP concentration was because the amount of initiation varied. The unexpected finding observed with GTP fixed at 0.4 mM was an apparent K_m value of 0.082 mM. This is considerably above that for polymerization and its significance will be discussed.

Summary of Apparent K_m Values with M. lysodeikticus DNA. In Table III a summary of the apparent K_m values and the conditions of the experiments is given. Values of approximately 0.15 mM considered to represent initiation are shown in expt 1a and 4. Values of approximately 0.015 mM which are considered to be related to polymerization are shown in expt 1b–d. These values are similar to those noted in Table II and to the value of 0.013 mM observed for UTP with

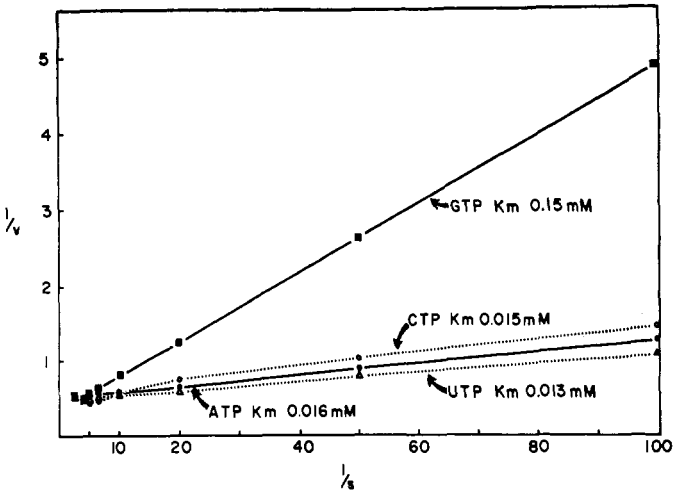

FIGURE 8: The effect of varying a single nucleoside triphosphate concentration on the kinetics of nucleotide incorporation with *M. lysodeikticus* DNA. Reaction volume, content, and incubation were as described in the legend to Figure 5, except that one nucleotide was varied and the concentration of the other three nucleotides was fixed at 0.4 mM. Either [^{3}H]CTP (1800 cpm/mμmole) or [^{3}H]-GTP (1300 cpm/mμmole) was used.

dAT. In expt 1b–d conditions for adequate initiation were probably present throughout the incubation period. In expt 2 and 3, in which there was prior initiation of RNA synthesis and washing of the DNA–RNA enzyme complex with 0.4 M $(NH_4)_2SO_4$, values of 0.027 and 0.030 mM were obtained. These values are slightly higher than other values considered to be related to polymerization. When GTP, the initiating nucleotide, was at high concentration and the other three varied, an intermediate value of 0.082 mM was found (Table III, expt 5). A similar value (0.098 mM) was observed with calf thymus DNA when ATP was fixed at 0.4 mM (Figure 1). The reason for these values is not clear.

Discussion

The reactions which are assumed to be involved in association, initiation, and polymerization by the RNA polymerase are indicated (steps 1–6).

association: DNA + enzyme $\rightleftharpoons$ DNA–enzyme (1)

initiation:

DNA–enzyme + XTP_1 $\rightleftharpoons$ DNA–enzyme–XTP_1 (2)

DNA–enzyme–XTP_1 + XTP_2 $\rightleftharpoons$ DNA–enzyme–XTP_1–XTP_2 (3)

DNA–enzyme–XTP_1–XTP_2 $\rightleftharpoons$ DNA–enzyme–XTP_1–XMP_2 + PP (4)

polymerization:

migration of enzyme on DNA (5)

DNA–enzyme–XTP_1–XMP_2 + XTP_3 $\rightleftharpoons$ DNA–enzyme–XTP_1–XMP_2–XMP_3 + PP (6)

Initiation as defined kinetically must be a combination of steps 2–4 since the binding of either XTP_1 or XTP_2 may be readily reversible. Step 4 may be reversible (Maitra and Hurwitz, 1967) but the concentration of inorganic pyrophosphate at the initiation of synthesis would be such that the direction of the reaction would be far toward phosphodiester bond formation. Only after formation of the first phosphodiester bond would the complex become more stabilized. Thus, the addition of the first nucleotide (step 2) would be observed by any method which measured RNA synthesis only by the addition of subsequent nucleotides (step 3, etc.). In the analysis of the kinetics presented below, it is assumed that initiation occurs at the beginning of the incubation period, in a time interval which is very short compared with the time for polymerization. This assumption is known to be inaccurate for nucleotide levels of 0.08 mM (Maitra and Hurwitz, 1965) with T2 DNA. In this case continuous initiation has been demonstrated, but in spite of this, the rate of nucleotide incorporation, which represents primarily polymerization, is linear during the first 10–15 min of the reaction. Thus, it is clear that the combination of initiation and polymerization is complex and the assumption of continuous initiation results in a model too involved for analysis with only this type of data. For this reason the values observed in these experiments, where as a

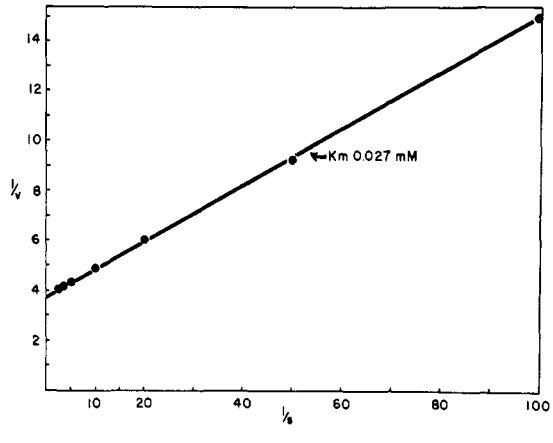

FIGURE 9: The effect of prior initiation on the kinetics of nucleotide incorporation with *M. lysodeikticus* DNA with a varied concentration of GTP. The preincubation and incubation were similar to those noted under Figure 7 except that the concentration of GTP was varied and the concentrations of ATP, UTP, and [^{3}H]CTP were kept constant at 0.4 mM.

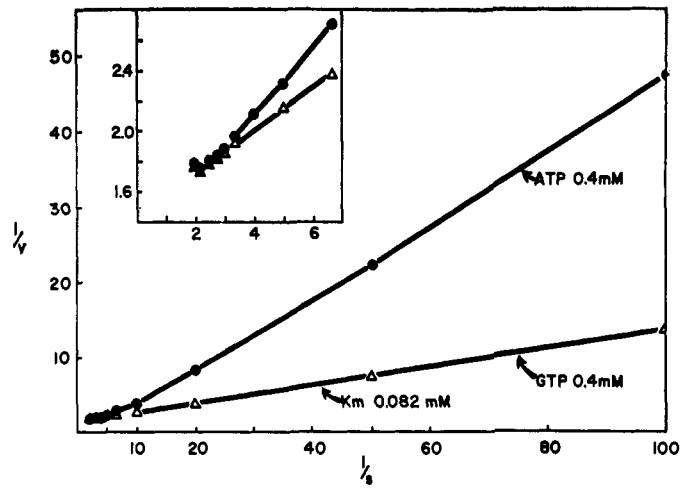

FIGURE 10: The effect of a high concentration (0.4 mM) of either GTP or ATP with variation of the concentration of the other three nucleoside triphosphates on the kinetics of [^{3}H]CMP incorporation with DNA of *M. lysodeikticus*. Reaction volume, contents, and incubation were as reported in the legend to Figure 5, except for the variation in concentration of the three nucleotides. The specific activity of [^{3}H]CTP was 8000 cpm/mμmole.

first approximation Michaelis–Menten kinetics are assumed to be applicable, are called apparent K_m values.

A nonlinear plot of the reciprocal of nucleotide incorporation *vs.* the reciprocal of nucleotide concentration when all four nucleotides are varied has been observed with calf thymus DNA (Figure 1), T4 DNA (Figure 3), *M. lysodeikticus* DNA (Figure 5), and dAT (Figure 4). This curvilinearity can be eliminated in two ways. The first is to fix one specific nucleotide at an elevated concentration (0.2–0.4 mM) while the other three are varied. This was observed with calf thymus DNA when ATP was fixed (Figure 1), *M. lysodeikticus* DNA when GTP was fixed (Figure 9), and dAT when ATP was fixed (Figure 4). The resulting linear plots were associated with an increased rate of nucleotide incorporation compared to the control. A relationship between the increase of incorporation produced by a specific nucleotide with a specific DNA correlated with observations of others (Maitra and Hurwitz, 1965; Maitra *et al.*, 1967) on the 5′-terminal nucleotide found in the RNA synthesized with that particular DNA. This suggested that the stimulation was related to initiation.

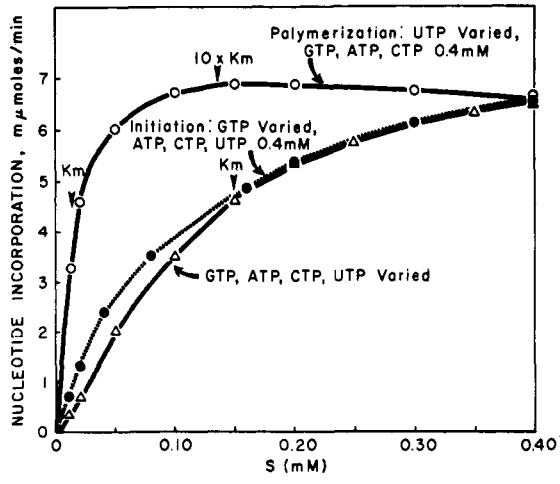

FIGURE 11: The effect of nucleotide concentration on the incorporation of nucleotides based on the high apparent K_m for initiation and the low apparent K_m for polymerization.

The second way to eliminate the curvilinearity was first to initiate synthesis of RNA by incubation with high concentrations of the four unlabeled nucleotides, then to filter the initiated DNA–enzyme–RNA complexes on nitrocellulose membranes and incubate with varying concentrations of nucleotides one of which was labeled. With T4 DNA (Figure 3), calf thymus DNA (not shown), and *M. lysodeikticus* DNA (Figure 7) linear plots were obtained. Thus, in these two types of experiments, the elimination of the curvilinear response was considered to be due to adequate initiation of RNA synthesis. Furthermore with calf thymus DNA, after prior initiation as above, there was no differential effect of ATP at 0.2 mM compared with CTP at 0.2 mM when the other three nucleotides were at 0.01 mM (Anthony *et al.*, 1966). This provided further evidence that the nucleotide effect was on initiation and not on polymerization.

The evidence that the effect of elevated concentrations of specific nucleotides, particularly purine nucleotides, was on the initiation process and not due to some allosteric effect is as follows. (1) A variability was observed with different DNAs (Table I). (2) There was a correspondence between the stimulating effect of nucleotides and the 5′-terminal RNA end groups (Maitra and Hurwitz, 1965). (3) Prior initiation changed the curvilinear double-reciprocal plot of velocity *vs.* nucleotide concentration to a linear plot with calf thymus, T4, and *M. lysodeikticus* DNA. (4) Prior initiation eliminated the differential stimulatory effect of ATP compared with CTP with calf thymus DNA (Anthony *et al.*, 1966), and altered the apparent K_m for GTP with *M. lysodeikticus* DNA. (5) With the polymer dI:dC, ATP did not stimulate the incorporation of CMP (Anthony *et al.*, 1966).

The experiments reported in this paper lead to the conclusion 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 was approximately 0.015 mM. Several types of experiments support this conclusion. With calf thymus DNA (with which the 5′-terminal

RNA nucleotides are primarily ATP and GTP), when three of the nucleotides were at 0.2 mM and the fourth varied, low values were obtained (Table II). The apparent K_m values with ATP and CTP were similar. In these experiments it is assumed that adequate initiation occurred with at least one of the purine nucleotides. Similar results were obtained with *M. lysodeikticus* DNA when ATP or CTP, or UTP was varied (Figure 8), and with dAT when UTP was varied (Figure 4). Finally prior initiation with *M. lysodeikticus* DNA and variation either of the four nucleotides or of GTP gave values of approximately 0.03 mM. This value is somewhat higher than 0.015 mM, but considerably lower than the values for initiation. Thus, in experiments in which conditions for adequate initiation are present, the lowest apparent K_m values obtained were in the range of 0.015 mM.

The apparent K_m for initiation is probably one order of magnitude higher than that for polymerization. This is most clearly demonstrated in the experiments with *M. lysodeikticus* DNA. Here over 85% of the RNA 5′-terminal end groups are GTP. When ATP, CTP, and UTP were held at 0.4 mM and GTP was varied, a linear double-reciprocal plot was observed and the apparent K_m for GTP was 0.15 mM (Figure 8). This apparent K_m for GTP of 0.15 mM was decreased to a value of 0.03 mM by prior initiation (Figure 9) which supports the conclusion that the higher value is related to initiation. The apparent K_m for GTP of 0.15 mM is similar to the value of 0.17 mM observed in the experiment in which the four nucleotides were varied. This value was calculated from the linear portion of Figure 5 and implies that in the concentration range above 0.1 mM with *M. lysodeikticus* DNA, initiation by GTP becomes the rate-determining step. From data (Maitra *et al.*, 1967; Figure 1) concerning the incorporation of the 5′-terminal GTP with *M. lysodeikticus* DNA when ^{32}P was measured as a function of the concentration of [γ-^{32}P]GTP, it is possible to calculate an apparent K_m value of 0.1 mM.

There are several possible explanations for the nonlinear double-reciprocal plots of nucleotide incorporation *vs.* nucleotide concentration. The first involves the observations which lead to the conclusion that the apparent K_m for the 5′-terminal nucleotide was approximately tenfold that for nucleotides involved in polymerization. A plot based on these two K_m values is shown in Figure 11. The curve labeled polymerization represents the relationship with *M. lysodeikticus* DNA between nucleotide incorporation and the concentration of UTP (from Figure 8). The other three nucleotides were held at 0.4 mM. The apparent K_m for UTP was 0.013 mM. At approximately ten times this concentration, a saturation level of UTP for polymerization is reached. (A slight inhibition at higher nucleotide levels was observed in this experiment and also in Figures 1 and 2.) The second curve is from the data (Figure 8) for GTP with a K_m of 0.15 mM and maximum velocity for nucleotide incorporation equivalent to that observed with UTP above. Data for [γ-^{32}P]GTP incorporation with *M. lysodeikticus* DNA (Maitra *et al.*, 1967; Figure 1) can be practically super-

TABLE III: Summary of Apparent K_m Values with *M. lysodeikticus* DNA.

Expt	Exptl Concn	Nucleotide Concentrations		Linear Reciprocal Plot in Concn Range (mM)	Presented	App K_m
		Varied	Constant (0.4 mM)			
1a	No preincubation	GTP	ATP, CTP, UTP	0.01–0.30	Figure 8	0.15
b	No preincubation	ATP	GTP, CTP, UTP	0.01–0.20	Figure 8	0.016
c	No preincubation	CTP	ATP, GTP, UTP	0.01–0.05	Figure 8	0.015
d	No preincubation	UTP	ATP, GTP, CTP	0.01–0.15	Figure 8	0.013
2	Preincubated, four nucleotides	GTP	ATP, CTP, UTP	0.01–0.20	Figure 9	0.027
3	Preincubated, four nucleotides	GTP, ATP, CTP, UTP		0.01–0.20	Figure 7	0.030
4	No preincubation	GTP, ATP, CTP, UTP		0.10–0.45	Figure 5	0.17
5	No preincubation	ATP, CTP, UTP	GTP	0.02–0.25	Figure 10	0.082

imposed on this curve. The third curve is that observed when all four nucleotides were varied. As is evident, above 0.15 mM, where the polymerization reaction is saturated, this curve approaches the Michaelis–Menten curve for initiation. This may explain why at nucleotide concentrations greater than 0.15 mM the kinetics were linear (Figure 5) and the n value of the Hill plot (Figure 6) was 1.0. Below 0.15 mM, varying the concentration of the four nucleotides affected both initiation and polymerization and a sigmoid curve, which in a double-reciprocal plot would be a parabola, was the result.

The second explanation for the nonlinear double-reciprocal plot is based on the requirement that the formation of the first phosphodiester bond involves two substrate molecules while subsequent nucleotides are added one by one. Curvilinear kinetics are observed when the concentration of two substrate molecules is varied and the reaction involves a random rapid equilibrium (Morrison and Cleland, 1966). A possibility which further complicates initiation is an apparent K_m for the second or subterminal nucleotide that may be higher that the K_m for polymerization. Data from some unpublished experiments with *M. lysodeikticus* DNA on the stimulatory effect of a high concentration of a second nucleotide besides GTP on the rate of incorporation can be explained in this way.[2]

A third explanation for the curvilinearity is the short lag in the rate of nucleotide incorporation noted at low concentrations of the four nucleotides. However, as indicated in the Results section, when rates are measured beyond the lag, the double-reciprocal plot is still nonlinear, so this explanation accounts for only part of the observed nonlinearity.

The reason for the short lag period at low nucleotide concentration is not clear. At low temperature or at high ionic strength, a lag has been observed which has been interpreted as a melting of the double-stranded DNA by the RNA polymerase (Walter *et al.*, 1967). In light of the conditions used for the experiments reported here, this explanation of the lag seems unlikely. Another possible explanation is that a fraction of the enzyme binds at some sites where initiation does not occur (Pettijohn and Kamiya, 1967). A high concentration of either purine nucleoside triphosphate (5 mM) has been shown to dissociate the DNA–enzyme complex at 5° (Anthony *et al.*, 1968). It is possible that at higher temperatures nucleotide concentrations of 0.2–0.4 mM will also dissociate rapidly the enzyme from an unproductive complex, while at 0.01 mM, this occurs more slowly. This explanation would also account for the continuous initiation described above. This explanation of the lag is at present only an hypothesis.

It should be emphasized that this kinetic separation of initiation from polymerization is the most simple interpretation of a very complex reaction sequence. Standard biochemical texts on kinetics do not describe any comparable enzyme systems in which the substrate concentrations required for initiation are different from those necessary for polymerization. Unexplained is the observation that with prior initiation the apparent K_m, when either all four nucleotides were varied (Table III, expt 3) or when GTP was varied (Table III, expt 2), was approximately 0.030 mM, a value somewhat higher than that for polymerization. Another unexplained high K_m was observed with *M. lysodeikticus* DNA with GTP at 0.4 mM (Table II, expt 5). This may be due to an elevated K_m for the second nucleotide, or it may be a K_m for residual initiation by CTP and ATP (Maitra *et al.*, 1967). Similarly, initiation by GTP may be the explanation for the high apparent K_m of 0.98 observed with calf thymus DNA and ATP at 0.4 mM (Figure 1).

It is recognized that there are assumptions and experimental difficulties not accounted for by this kinetic model with the different apparent K_m's for initiation and polymerization. Therefore this model must be taken as a first approximation to a more rigorous definition of initiation and polymerization which must arise from other approaches to the problem.

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