Studies on the Kinetics of Ribonucleic Acid Chain Initiation and Elongation*

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ABSTRACT: The effect of the ribonucleoside triphosphate concentration on ribonucleic acid (RNA) chain initiation and elongation has been studied using poly d(A-T) copolymer and the RNA polymerase of *Escherichia coli*. A sigmoid curve was observed when the rate of chain initiation was measured as a function of ribonucleoside triphosphate concentration. Normal Michaelis–Menten kinetics were obtained when either the adenosine triphosphate (ATP) or the uridine triphosphate (UTP) concentration was saturating or when the initiation step was bypassed by preincubation of the poly d(A-T)–enzyme complex with the complementary dinucleotides ApU or UpA. The results indicate that chain initiation is a bimolecular reaction and the rate-limiting step in the synthesis of RNA. The apparent K_m's for ATP and UTP were found to be 2.5×10^{-5} M, as measured either for initiation or elongation. Although the apparent K_m's for the initiating ribonucleoside triphosphate and those involved in chain elongation are identical, chain initiation was found to be more sensitive to fluctuations in substrate concentration than was chain elongation. This is explained on the basis that initiation is a second-order reaction with respect to substrate, while chain elongation is a first-order reaction.

Complex kinetics have been observed with the DNA-dependent RNA polymerase when the rate of RNA synthesis is measured as a function of nucleotide concentration. The double-reciprocal plot of initial velocity *vs.* substrate concentration is nonlinear when either native DNA or a synthetic polynucleotide is used as template (Niyogi and Stevens, 1965; Anthony *et al.*, 1969).

Studies on chain initiation and elongation, the two sequential steps involved in RNA synthesis, have shown that the direction of chain growth is from the 5′-hydroxyl end to the 3′-hydroxyl end, and that the 5′-terminal ribonucleoside triphosphate is most frequently a purine nucleotide (Bremer *et al.*, 1965; Maitra and Hurwitz, 1965). Others have suggested that initiation of the RNA chain, or the formation of the first phosphodiester bond, is the rate-limiting step in the synthesis of RNA (Niyogi and Stevens, 1965; Anthony *et al.*, 1969).

Kinetic studies of RNA synthesis are complicated by the fact that the formation of the first phosphodiester bond, as well as the addition of subsequent ribonucleoside triphosphates to the 3′-hydroxyl end of the growing RNA chain, is dictated by the base sequence in the DNA template, as yet unknown for DNAs of mammalian or microbial origin. Thus, a kinetic analysis of a sequential reaction requiring four substrates with these DNAs is rather difficult.

For these reasons the synthesis of poly r(A-U) with poly d(A-T)[1] copolymer has been chosen as a model system to study the kinetics of RNA synthesis. The poly d(A-T) copolymer is a double-helical DNA containing deoxyadenylate and thymidylate in alternating sequence (Schachman *et al.*, 1960). Maitra and Hurwitz (1965) showed that initiation with this DNA occurs almost exclusively with ATP. An analysis of the kinetics of RNA chain initiation and elongation with this system is presented.

Experimental Procedures

DNA-dependent RNA polymerase was isolated from *Escherichia coli* B cells by the method previously described (So *et al.*, 1967). [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (1964).

Polymerization Assay. The reaction mixture contained 0.08 M Tris-HCl buffer (pH 7.8), 10 mM $MgCl_2$, 4.8 mM β-mercaptoethanol, 0.08 absorbance unit of poly d(A-T), 2.5–5 μg of enzyme, ATP, and UTP (one labeled with ^{3}H) in a final volume of 0.25 ml. The ribonucleoside triphosphate solutions were diluted from a stock solution immediately before use. The reaction mixture was preincubated for 4 min at 37° and the reaction was started by the addition of enzyme. After incubating for 5 min, the reaction was stopped by the addition of cold 5% trichloroacetic acid. The precipitate was collected on a glass filter paper (Whatman GF/C) and washed with cold trichloroacetic acid and ethanol. The filter was dried and counted in a Tri-Carb liquid scintillation counter.

Initiation Assay. The reaction mixture was the same as described for the polymerization assay except that [γ-^{32}P]ATP was used instead of [^{3}H]ATP, and the 5% trichloroacetic acid contained 0.01 M PP_i.

[^{3}H]ATP and [^{3}H]UTP were obtained from Schwarz BioResearch, Inc.; [^{32}P]phosphoric acid was purchased from New England Nuclear Corp. Unlabeled ribonucleoside triphosphates were from P-L Biochemicals, Inc., and poly d(A-T) was obtained from Biopolymers Laboratory, General

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[1] The following abbreviations have been used: poly d(A-T), alternating copolymer of deoxyadenylate and thymidylate; poly r(A-U), alternating copolymer of adenylate and uridylate.

TABLE I: Stimulation of RNA Synthesis by either ATP or UTP.[a]

Ribonucleoside Triphosphate Added	Concn (μM)	% Stimulation
ATP	4	Control
	8	38
	12	65
	20	109
	36	152
UTP	4	Control
	8	48
	12	73
	20	103
	36	135

[a] The reaction mixtures were essentially as described in Experimental Procedures except (1) when the ATP concentration was varied, the [^{3}H]UTP concentration was held constant at 4 μM, and (2) when the UTP concentration was varied, the [^{3}H]ATP concentration was held constant at 4 μM.

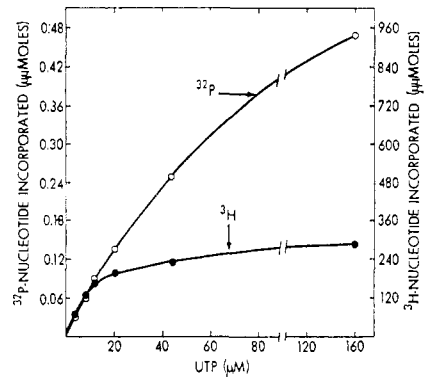

FIGURE 1: Effect of UTP on initiation and polymerization. The reaction mixtures were as described in Experimental Procedures except (1) the ATP concentration was held at 4 μM and was labeled either with γ-^{32}P or ^{3}H, (2) the UTP concentration was varied from 4 to 160 μM, and (3) the reaction mixture was incubated for 7 min at 37°.

Biochemicals. *E. coli* B (grown in enriched media, 0.5 log) was purchased from Grain Processing Corp. ApU, UpA, and other dinucleotides were obtained from Sigma Chemical Co. Muscle glyceraldehyde 3-phosphate dehydrogenase, 3-phosphoglycerate, und yeast phosphoglycerate kinase were obtained from Boehringer und Soehne, G.m.b.H.

Results

Effect of Nucleotide Concentration on RNA Synthesis with d(A-T) Copolymer. Although the synthesis of RNA with d(A-T) copolymer as a template requires the presence of both ATP and UTP, either ATP or UTP alone can stimulate poly r(A-U) synthesis when the alternate ribonucleoside triphosphate is held constant at a low concentration. This effect is shown in Table I, where it can be seen that increasing ATP concentrations stimulate RNA synthesis when UTP is held constant at 4 μM and that increasing UTP concentrations yield similar results when ATP is held constant at 4 μM. The amounts of stimulation of RNA synthesis by ATP and UTP are identical. Control experiments have shown that synthesis of poly A or U does not occur under these conditions. The noncomplementary nucleotides, ITP, GTP, and CTP, had no effect on the synthesis of poly r(A-U).

Effects of UTP Concentration on Initiation and Polymerization. The effects of increasing UTP concentration on chain initiation and polymerization are shown in Figure 1, where the incorporation of [γ-^{32}P]ATP is used as a measure of initiation and the incorporation of [^{3}H]ATP is used as a measure of polymerization. In these experiments the concentration of either [γ-^{32}P]ATP or [^{3}H]ATP was held constant at 4 μM, while the concentration of UTP was varied from 4 to 160 μM. Parallel increases in the rates of chain initiation and elongation resulted when the UTP concentration was increased from 4 to 12 μM. However, further increases in the UTP concentration stimulated the rate of chain initiation to a greater extent than the rate of chain elongation, with a resultant marked decrease in the average chain length of the RNA synthesized. The average chain length at 4 μM UTP was approximately 2200 nucleotides while at 160 μM, the average RNA chain was only 600 nucleotides long. Similar results were obtained when the ATP concentration was increased while the UTP concentration was held constant at 4 μM. However, when the concentration of both ATP and UTP was increased simultaneously, the average chain length of the RNA synthesized remained constant. It should be emphasized that it is not the ultimate length of the RNA chains that is being reported, but rather the average chain length that has been calculated from measurements of initial rates. It is apparent from these results that chain initiation is more sensitive than chain elongation to fluctuations in the substrate levels.

Effect of Ribonucleoside Triphosphate Concentration on the Kinetics of RNA Synthesis. As has been observed by many investigators, a sigmoid curve is obtained when the rate of RNA synthesis is plotted as a function of nucleotide concentration (Niyogi and Stevens, 1965; Anthony *et al.*, 1966, 1969). A similar effect is observed with d(A-T) copolymer as a template. The effect of ATP and UTP concentration on the rate of RNA synthesis is shown in Figure 2. The insert shows the double-reciprocal plot, $1/V$ *vs.* $1/S$, which is concave upward. When the concentration of either ATP or UTP is saturating while the concentration of the alternate ribonucleoside triphosphate is varied, normal Michaelis–Menten kinetics are observed, as shown in Figures 3 and 4. In Figure 3 the ATP concentration is held constant at 0.4 mM, and the UTP concentration is varied from 2 to 32 μM. The resulting double-reciprocal plot becomes linear under these conditions, and the apparent K_m obtained for UTP is approximately 2.5×10^{-5} M. The opposite experiment is shown in Figure 4, where UTP is held constant at 0.4 mM, and the ATP concentration is varied. As can be seen, the effect of holding the UTP concentration high is similar to the effect of saturating ATP in the reaction mixture, and the apparent K_m for ATP is also 2.5×10^{-5} M. Thus, the apparent K_m's for ATP and UTP are identical.

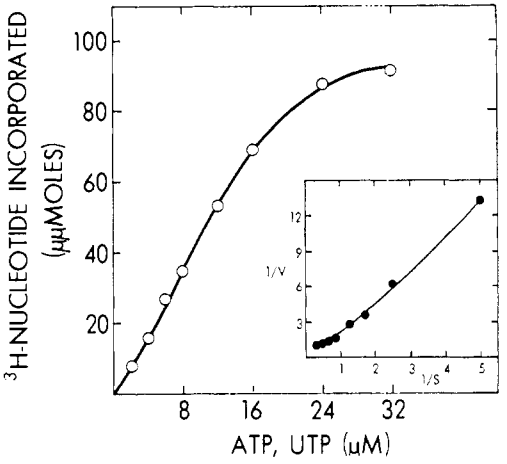

FIGURE 2: Effect of ribonucleoside triphosphate concentration on the rate of RNA synthesis. The reaction mixtures were as described in Experimental Procedures, except that both [^{3}H]ATP and UTP concentrations were varied from 2 to 32 μM. The enzyme concentration was 1.4 μg in a final volume of 0.25 ml.

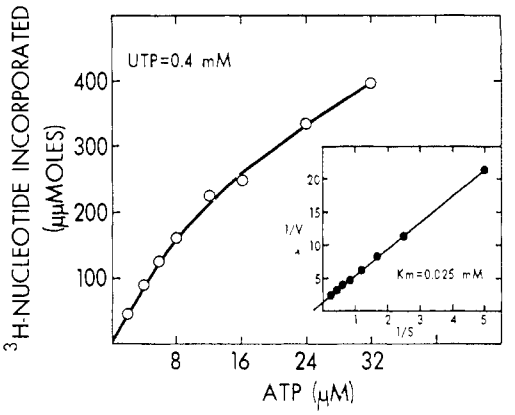

FIGURE 4: Effect of UTP concentration on the kinetics of poly r(A-U). Synthesis at low enzyme concentration. The assay conditions were as described in Figure 2 except that the UTP concentration was held constant at 0.4 mM and the [^{3}H]ATP concentration was varied from 2 to 32 μM. The enzyme concentration was 2.8 μg in a final volume of 0.25 ml.

Effect of Nucleotide Concentration on the Kinetics of Chain Initiation. The rate of chain initiation as a function of ribonucleoside triphosphate concentration is shown in Figure 5, where the incorporation of [γ-^{32}P]ATP is used as a measure of chain initiation. The sigmoid curve that results when the rate of total RNA synthesis is plotted as a function of ribonucleoside triphosphate concentration (Figure 2) is even more pronounced when the rate of RNA initiation is measured (Figure 5). The double-reciprocal plot (insert), $1/V$ *vs.* $1/S^2$, is linear. This is consistent with the hypothesis that chain initiation is a bimolecular reaction, *i.e.*, that the d(A-T)–enzyme complex binds two molecules of substrate for each chain initiated.

As is the case for total RNA synthesis, saturating concentrations of either ATP or UTP relieve the lag in initiation observed at low ribonucleoside triphosphate concentrations, and the double-reciprocal plot, $1/V$ *vs.* $1/S$, becomes linear.

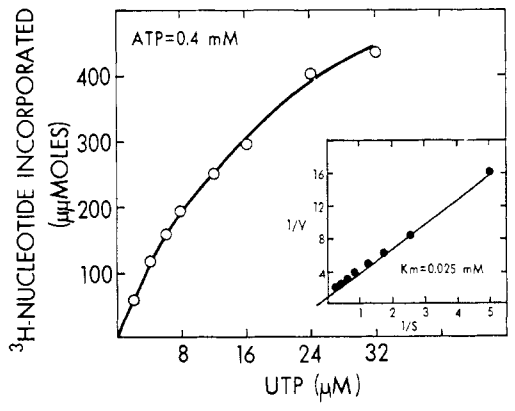

FIGURE 3: Effect of ATP concentration on the kinetics of poly r(A-U) synthesis at low enzyme concentration. The assay conditions were as described in Figure 2 except that the ATP concentration was held constant at 0.4 mM and the [^{3}H]UTP concentration was varied from 2 to 32 μM. The enzyme concentration was 2.9 μg in a final volume of 0.25 ml.

The effect of saturating levels of UTP on the kinetics of initiation is shown in Figure 6. The apparent K_m for ATP, as measured by initiation, was found to be approximately 3×10^{-5} M, very similar to the K_m's for ATP and UTP determined by polymerization assays. These results support the suggestion that the formation of the first phosphodiester bond is a bimolecular reaction.

Kinetics of RNA Synthesis at High Enzyme Concentration. The effect of UTP concentration on the rate synthesis at high enzyme concentration (Figure 7) differs from that observed at low enzyme concentration (Figure 3). As can be seen in Figure 7, at high enzyme concentration, a sigmoid curve with a nonlinear double-reciprocal plot, $1/V$ *vs.* 1/(UTP), was observed even in the presence of 0.4 mM ATP. This experiment is identical with that shown in Figure 3, except

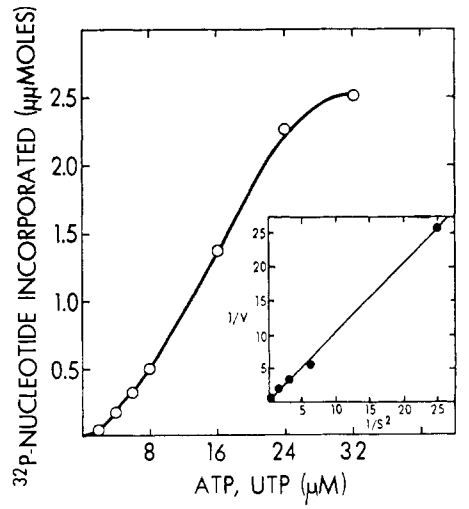

FIGURE 5: Effect of ribonucleoside triphosphate concentration on the kinetics of initiation. The assay conditions were as described in Figure 2 except that the ATP was labeled with ^{32}P in the γ position and the concentration of enzyme was 5.3 μg in a final volume of 0.25 ml.

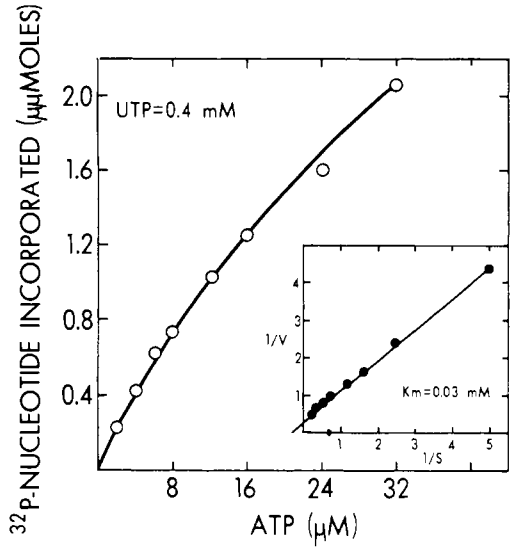

FIGURE 6: Effect of UTP concentration on the kinetics of initiation. The incubation conditions were as described in Figure 4 except that [γ-^{32}P]ATP was used instead of [^{3}H]ATP and the enzyme concentration was 5.3 μg in a final volume of 0.25 ml.

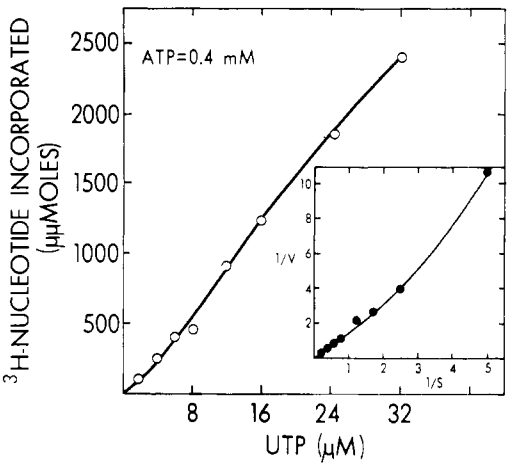

FIGURE 7: Effect of UTP concentration on the kinetics of poly r(A-U) synthesis at high enzyme concentration. The incubation conditions were as described in Figure 3 except that the enzyme concentration was 23 μg in a final volume of 0.25 ml.

that the enzyme concentration is approximately eight times higher. It is apparent that the ATP concentration that was sufficient to saturate the system at low enzyme concentration is no longer saturating at higher enzyme concentrations. Thus, the concentration of substrate needed to saturate the system is dependent on the number of chains initiated and this, in turn, is dependent on the enzyme concentration.

That increasing the enzyme concentration does increase initiation relative to elongation is shown in Table II. This Table shows the effect of increasing enzyme concentration on both chain initiation and polymerization under conditions of limiting substrate. It can be seen that increasing enzyme concentrations lead to increasing rates of both initiation and polymerization, although the effect on the rate of chain initiation is more marked than the effect on the rate of polymerization. This results in a progressive decrease in the average RNA chain length. Similar results are obtained when one of the substrates is saturating. However, when both ATP and UTP are saturating, both initiation and polymerization increase proportionally as the enzyme concentration is increased and this is reflected in a constant average chain length. Thus, when the supply of substrate is limiting, competition exists between chain initiation and elongation for the available ribonucleoside triphosphates.

Effects of ApU and UpA on Chain Initiation and Elongation. In order to measure the rate of chain elongation independently of initiation, the complementary dinucleotides ApU and UpA were used as preformed RNA chains. In this way, the initiation step could be bypassed. The effects of the dinucleotide UpA on chain initiation and elongation are shown in Figure 8. It can be seen that increasing concentrations of UpA markedly inhibited the incorporation of [γ-^{32}P]ATP, but greatly stimulated the synthesis of poly r(A-U). Identical results were obtained with the other dinucleotide complementary to poly d(A-T), ApU. These results would suggest that the dinucleotides ApU and UpA are functioning as preformed chains and are competing with [γ-^{32}P]ATP for initiation sites on the poly d(A-T)–enzyme complex. The

TABLE II: Effect of Enzyme Concentration on the Average RNA Chain Length.[a]

Enzyme Concn (μg)	[γ-^{32}P]ATP Incorp (μμmoles)	Total RNA Synthesized (μμmoles)	Av Chain Length
1.25	0.12	108	1858
2.50	0.29	158	1086
5.00	0.50	247	988
10.00	1.08	354	656
15.00	1.89	437	462
20.00	3.49	528	302

[a] The reaction mixtures were as described in Table I except that the concentrations of ATP and UTP were held constant at 8 μM, and ATP was labeled either with γ-^{32}P or ^{3}H. The concentration of enzyme was varied as indicated.

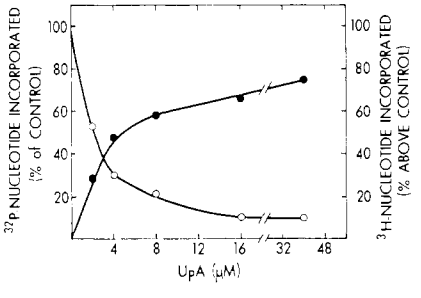

FIGURE 8: Effect of UpA on chain initiation and polymerization. The reaction mixtures were as described in Experimental Procedures except that (1) the ATP and UTP concentrations were held constant at 16 μM and the concentration of UpA was varied as indicated and (2) the mixtures were incubated for 10 min at 37°. The enzyme concentration was 10 μg in a final volume of 0.25 ml.

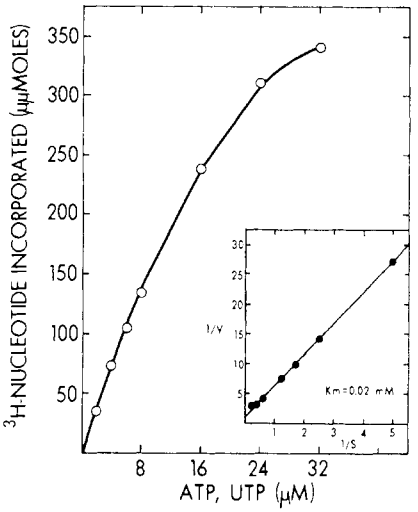

FIGURE 9: Effect of ApU on the kinetics of RNA synthesis. The reaction conditions were as described in Figure 2, except that the reaction mixtures were preincubated in the absence of ribonucleoside triphosphates with 0.16 mM ApU for 2 min at 37°. The reaction was started by the addition of [^{3}H]ATP and UTP.

results further support the suggestion that chain initiation is the rate-limiting step in the synthesis of RNA, since the rate of poly r(A-U) synthesis is stimulated when the initiation step is bypassed. Control experiments have shown that noncomplementary dinucleotides such as GpA, UpC, UpG, ApA, UpU, and ApG have no effect on the synthesis of poly r(A-U). These results are consistent with the observations of Niyogi and Stevens (1965). Using the homopolymers poly rA and poly rU as templates for the synthesis of the complementary homopolymers, these investigators found complementary oligonucleotides could act as chain initiators and were incorporated into the 5′ ends of the polynucleotides synthesized. The stimulatory effect of dinucleotides on the synthesis of RNA with *E. coli* DNA as template have also been reported by Gros *et al.* (1963).

Studies on Kinetics of Chain Elongation. The effects of nucleotide concentration on the kinetics of chain elongation are shown in Figure 9. In this experiment, the dinucleotide ApU is used as a preformed chain and the incorporation of [^{3}H]nucleotide measures the rate of chain elongation alone. Normal Michaelis–Menten kinetics are observed, even at low substrate concentrations, when the rate of chain elongation is measured as a function of ribonucleoside triphosphate concentration. The apparent K_m calculated for ATP and UTP was approximately 2.0×10^{-5} M. Similar results were obtained when UpA was used to bypass the initiation step. Thus, the rate-limiting step in the synthesis of RNA is the formation of the first phosphodiester bond.

Discussion

The present study demonstrates that chain initiation is a bimolecular reaction, and that the formation of the first phosphodiester bond is the rate-limiting step in the synthesis of RNA.

Kinetic studies using [^{3}H]nucleotide incorporation to measure the rate of RNA synthesis necessarily measure chain initiation as well as chain elongation. Although under limiting conditions, the process that is rate limiting becomes the rate-determining step in the synthesis of RNA, it is not possible to determine which step is rate determining (*i.e.*, chain initiation or chain elongation) by measuring only the rate of incorporation of [^{3}H]nucleotide.

We have used [γ-^{32}P]ATP incorporation to study the kinetics of RNA chain initiation, as this measures only the incorporation of nucleotide into the 5′ end of the RNA chain. However, it should be pointed out that although [γ-^{32}P]ATP incorporation measures the rate of chain initiation, sufficient polymerization of nucleotides must occur before the chain is acid precipitable.

We have also used dinucleotides complementary to the d(A-T) copolymer to study the kinetics of RNA synthesis. These dinucleotides (ApU and UpA) can function as performed chains and allow direct measurement of the rate of chain elongation by bypassing the initiation step. By the use of these three measurements, some insight into the kinetics of RNA synthesis has been gained.

It has been known for some time that the rate of RNA synthesis is some exponential function of the substrate concentration, and several explanations have been offered to account for the kinetic behavior of the enzyme (Richardson, 1969). Perhaps the most intriguing explanation is that RNA polymerase is an allosteric protein with the substrates acting as activators. In the present study, the kinetics of RNA synthesis may be adequately explained by the observation that chain initiation is a bimolecular reaction, *i.e.*, the formation of the first phosphodiester bond requires the binding of two molecules of substrate to the DNA–enzyme complex. However, the possibility that RNA polymerase is a regulatory enzyme has not been ruled out.

It has been suggested that the kinetic behavior of RNA polymerase may be explained by a marked difference in the K_m's for the initiating nucleotide and those involved in chain elongation (Anthony *et al.*, 1969). These investigators have reported that the apparent K_m for the 5′-terminal ribonucleoside triphosphate is 10-fold higher than the apparent K_m for the ribonucleoside triphosphates incorporated into internal parts of the RNA chain.

In our studies with d(A-T) copolymer as template, no difference in the apparent K_m's for ATP and UTP has been found. The apparent K_m's for ATP and UTP were found to be identical as determined by three separate methods: (1) by studying the kinetics of RNA chain initiation with [γ-^{32}P]-ATP; (2) by directly measuring the rate of chain elongation with [^{3}H]nucleotides in the presence of the dinucleotides ApU and UpA; and (3) by measuring the rate of RNA synthesis with [^{3}H]ATP and [^{3}H]UTP. The reason for the discrepancies between our findings and those reported by Anthony *et al.* (1969) are not apparent at present.

Although the apparent K_m's for the initiating ribonucleoside triphosphate and those involved in chain elongation are identical, chain initiation was found to be more sensitive to fluctuations in substrate concentration than was chain elongation. This is explained on the basis that initiation, or the formation of the first phosphodiester bond, is a second-order reaction with respect to substrate, while chain elongation, or the sequential addition of ribonucleoside triphosphates to the 3′ end of the nascent RNA chain, is a first-order reaction.

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Spin-Labeling Studies of Aminoacyl Transfer Ribonucleic Acid*

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ABSTRACT: Spin-labeling experiments have been carried out in which an organic free radical is linked to *Escherichia coli* Val- or Phe-tRNA by acylation of the α-amino group.

The chemistry of the labeling reaction is studied in detail, and special attention is paid to the specificity of the reaction. Nonspecific labeling of tRNA can be decreased considerably by fractionating the tRNA after the spin-labeling reaction. During this process, a single peak of spin-labeled Val-tRNA and two peaks of Phe-tRNA were recovered. The two species of Phe-tRNA differ by their stability to heat denaturation. A change in mobility of the spin label occurs after ribonuclease treatment and after thermal denaturation. The latter process is reversible, and the spin-melting temperature measured in this process is sensitive to ionic strength. The spin-melting phenomenon is interpreted as indicating two molecular states of tRNA with different activation energies for motion of the spin label at temperatures, respectively, above and below the sharp transition which occurs at the spin-melting temperature.

Spin-labeling is a technique in which a stable organic free radical linked to a macromolecule is used to provide information about the structure and function of the latter. Changes in macromolecular conformation can be observed through their effect on the rotational motion of the label. This technique has been used to study a variety of macromolecules (Stone *et al.*, 1965; Ogawa and McConnell, 1967; Smith, 1968; see also the review by Hamilton and McConnell, 1968), and in particular it has recently been extended to the study of nucleic acids (Smith and Yamane, 1967; Hoffman *et al.*, 1969). As with all methods involving the introduction of chemical probes, interpretation of the data is critically dependent on a knowledge of the site of attachment of the probe. We have applied the spin-labeling technique to study tRNA paying particular attention to the specific labeling of selected sites in a fashion designed to minimize possible interference with the natural state of tRNA.

tRNA contains a relatively high proportion of unusual bases (Miura, 1967) such as N-methylated purines, dihydrouracil, 2- and 4-thiouracil, pseudouridine, and inosine. At first sight, these might seem attractive targets for selective labeling. However, there are very few chemical reagents that will discriminate among these bases to a sufficiently high degree. We have therefore chosen the α-amino group of AA-tRNA as a unique point of attack. This amino group with a pK of 8–9 is strongly basic in contrast to the amino groups and heterocyclic nitrogen atoms of the tRNA bases. Thus a selective chemical attack is possible. An unfractionated mixture of tRNAs can be charged enzymatically with a single amino acid and a spin label can then be attached chemically through an amide linkage to the α-amino group of the aminoacyl ester. The specificity of aminoacylation allows us to investigate only those tRNA species specific for the particular amino acid. This procedure is also less likely to perturb the tRNA structure than labeling one of the

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