Porubcan, M. A., Neves, D. E., Rausch, S. K., & Markley, J. L. (1978) *Biochemistry 17*, 4640–4647.
Reeck, G. R., Nelson, T. B., Paukstelis, J. V., & Mueller, D. D. (1977) *Biochem. Biophys. Res. Commun. 74*, 643–649.
Robertus, J. D., Kraut, J., Alden, R. A., & Birktoft, J. J. (1972) *Biochemistry 11*, 4293–4303.
Robinson, N. C., Neurath, H., & Walsh, K. A. (1973) *Biochemistry 12*, 420–426.
Santini, R. E., & Grutzner, J. B. (1976a) *Anal. Chem. 48*, 941–943.
Santini, R. E., & Grutzner, J. B. (1976b) *J. Magn. Reson. 22*, 155–160.
Schaffer, N. K., May, S. C., Jr., & Summerson, W. H. (1953) *J. Biol. Chem. 202*, 67–76.
Stroud, R. M., Kay, L. M., & Dickerson, R. E. (1974) *J. Mol. Biol. 83*, 185–208.
Westler, W. M., & Markley, J. L. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 1795.
Whitaker, D. R., & Roy, C. (1967) *Can. J. Biochem. 45*, 911–916.
Wilcox, P. E. (1970) *Methods Enzymol. 19*, 64–108.

Mechanistic Studies on Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerase from *Escherichia coli* Using Phosphorothioate Analogues. 1. Initiation and Pyrophosphate Exchange Reactions†

David Yee, Victor William Armstrong, and Fritz Eckstein*

ABSTRACT: The diastereomers of adenosine 5′-*O*-(1-thiotriphosphate) (ATPαS) and adenosine 5′-*O*-(2-thiotriphosphate) (ATPβS) can replace adenosine triphosphate (ATP) in the initiation reaction catalyzed by deoxyribonucleic acid (DNA) dependent ribonucleic acid (RNA) polymerase from *Escherichia coli*. In both cases, the S_p diastereomer is a better initiator than the R_p isomer. The diasteromers of 3′-uridyl 5′-adenosyl *O,O*-phosphorothioate [Up(S)A] can replace UpA in the primed initiation reaction catalyzed by RNA polymerase; however, the R_p diastereomer is a better initiator than the S_p isomer. By using ATP or CpA as initiator and UTPαS, isomer A, as substrate, we determined the stereochemical courses of both the initiation and primed initiation reactions, respectively, with T7 DNA template and found them to proceed with inversion of configuration. Determination of the stereochemical course of the pyrophosphate exchange reaction catalyzed by RNA polymerase provides evidence that this reaction is the reverse of the phosphodiester bond-forming reaction.

DNA-dependent RNA polymerase from *Escherichia coli* mediates the transcription of DNA to RNA by catalyzing the polymerization of ribonucleoside triphosphates in the presence of DNA template (Chamberlin, 1976). The DNA-directed synthesis of RNA by RNA polymerase may be considered to involve two kinds of phosphodiester bond-forming steps (Chamberlin, 1976; Krakow et al., 1976): (1) an initiation step, wherein a purine ribonucleoside triphosphate and another ribonucleoside triphosphate are coupled to give a dinucleoside tetraphosphate, and (2) an elongation step, wherein a ribonucleoside triphosphate is added to the 3′-OH terminus of the growing RNA chain. RNA polymerase can also catalyze a primed initiation reaction involving the addition of a ribonucleoside triphosphate to a dinucleoside monophosphate "primer" (So & Downey, 1970). Furthermore, RNA polymerase catalyzes a template-dependent pyrophosphate exchange into ribonucleoside triphosphates in the presence of an initiating ribonucleoside triphosphate or primer (Furth et al., 1962; Krakow & Fronk, 1969; So & Downey, 1970).

Phosphorothioate analogues of nucleoside triphosphates have proved to be useful in the study of enzyme mechanisms (Eckstein, 1975, 1979); e.g., the stereochemistry of the elongation step catalyzed by RNA polymerase has been determined by the use of ATPαS[1] (Eckstein et al., 1976; Burgers & Eckstein, 1978).

This paper reports on the substrate specificity and stereochemistry of the initiation, primed initiation, and pyrophosphate exchange reactions catalyzed by RNA polymerase using phosphorothioate analogues. The following paper (Armstrong et al., 1979) deals with the substrate specificity of the elongation step.

Experimental Procedure

Materials. ATP, UTP, UpA, and CpA were purchased from Pharma-Waldorf; poly[d(A-T)] was obtained from Miles Laboratories; ^{32}P-labeled Na_4PP_i was supplied by Amersham Buchler. Bacteriophage T7 DNA was prepared according to the procedure of Thomas & Abelson (1966), and T4 DNA was a generous gift from Professor W. Zillig. The molarities

† From the Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, D-3400 Göttingen, Federal Republic of Germany. *Received February 22, 1979.* This research was supported in part by a grant from the Deutsche Forschungsgemeinschaft.

[1] Abbreviations used: ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); ATPβS, adenosine 5′-*O*-(2-thiotriphosphate); UTPαS, uridine 5′-*O*-(1-thiotriphosphate); ADPαS, adenosine 5′-*O*-(1-thiodiphosphate); ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); UDPαS, uridine 5′-*O*-(1-thiodiphosphate; Up(S)A, 3′-uridyl 5′-adenosyl *O,O*-phosphorothioate; poly-[d(A-T)], copolymer of alternating deoxyadenylate and thymidylate; LC, high-pressure liquid chromatography.

0006-2960/79/0418-4116$01.00/0

of the phage DNA solutions were calculated by assuming a value of 0.020 $cm^2/\mu g$ for the specific absorbance of DNA at 260 nm (Abelson & Thomas, 1966) and a value of 25×10^6 and 110×10^6 for the molecular weight of T7 and T4 DNA, respectively (Freifelder, 1970). RNA polymerase holoenzyme was purified by the method of Sternbach et al. (1975) and generously donated by Dr. H. Sternbach. Snake venom phosphodiesterase was obtained from Boehringer Mannheim.

The diasteromers of ATPαS were prepared essentially as described by Eckstein & Goody (1976). A chemically synthesized mixture of ADPαS isomers was phosphorylated with pyruvate kinase to yield the A isomer of ATPαS. The unphosphorylated ADPαS (mainly isomer B) from this reaction was then phosphorylated with creatine kinase to yield ATPαS, isomer B, which was purified of contaminating A isomer by limited digestion with hexokinase (Stahl et al., 1974; Midelfort & Sarton-Miller, 1978). Similarly, the A isomer of UTPαS was prepared from a chemically synthesized mixture of UDPαS by phosphorylation with pyruvate kinase, the unreacted UDPαS from this reaction then being phosphorylated with creatine kinase to yield UTPαS, isomer B. This was purified of contaminating A isomer by incubation with nucleoside diphosphate kinase under the conditions described for ATPαS (Eckstein & Goody, 1976), except that the incubation time was increased to ~7 h.

ATPβS, isomer A, was synthesized from ADPβS by phosphorylation with pyruvate kinase (Eckstein & Goody, 1976). The product, which contained significant amounts of the B isomer, was purified by incubation with hexokinase in the presence of magnesium (Jaffe & Cohn, 1978). ATPβS, isomer B, was synthesized from ADPβS by phosphorylation with acetate kinase and purified of A isomer by incubation with myosin in the presence of calcium (Eckstein & Goody, 1976).

The diasteromers of Up(S)A were prepared as described by Burgers & Eckstein (1979) and generously donated by Dr. P. M. J. Burgers.

Initiation Assay. In the initiation assay reaction (see Table I for details) the enzyme was preincubated with the DNA template at 37 °C for at least 10 min before addition of substrates. The reaction was then allowed to proceed at 37 °C and monitored by LC, using a column (2 mm × 40 cm) of Nucleosil 10SB with buffer B (0.5 M KCl–0.05 M KH_2PO_4, pH 4.5) as described by Burgers & Eckstein (1978).

The major products of the initiation assay reactions were isolated by chromatographing the reaction mixture through a column of DEAE-Sephadex A-25 at 4 °C, using a linear gradient of triethylammonium bicarbonate (0.05–1.0 M, pH 8). The isolated products were further characterized by incubation at 37 °C with snake venom phosphodiesterase in a solution (total volume 0.3 mL) containing 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 0.3 mg/mL enzyme. These reactions were again monitored by LC.

Pyrophosphate Exchange Reaction. The pyrophosphate exchange reaction consisted of a solution (total volume 0.2 mL) containing 40 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 2.5 mM $Na_4[^{32}P]PP_i$, and varying amounts of initiator, substrate, RNA polymerase, and DNA template as described in the legends to the figures and tables. The reaction was assayed by determining the amount of charcoal-adsorbable radioactivity (Krakow & Fronk, 1969). After incubation of the reaction mixture at 37 °C for the specified time, a 30-μL aliquot was quenched with 0.1 mL of 0.3 M EDTA, 0.1 mL of saturated Na_4PP_i (adjusted to pH 6 with KH_2PO_4), 0.5 mL of 10% charcoal suspension in 0.01 M Na_4PP_i (adjusted to pH 6 with KH_2PO_4), and 3 mL of 0.01 M Na_4PP (pH 6). This mixture was then filtered onto a Whatman GF/C disk (presoaked in 0.01 M Na_4PP_i, pH 6) and washed with 0.01 M Na_4PP_i, pH 6, and water. The disk was placed, charcoal-side down, in a scintillation vial, dried, and counted in a toluene-based scintillation fluid with a Packard Tri-Carb liquid scintillation spectrometer.

Table I: Initial Rates for Formation of First Phosphodiester Bond[a]

initiator	v_i (mmol min^{-1} (μg of enzyme)$^{-1}$) with poly-[d(A-T)] template	v_i (mmol min^{-1} (μmol of DNA)$^{-1}$) with T4 DNA template	v_i (mmol min^{-1} (μmol of DNA)$^{-1}$) with T7 DNA
ATP			1.2
ATPαS, A		0.33	0.34
ATPαS, B		0.11	0.045
ATPβS, A			0.58
ATPβS, B			0.24
UpA	3.0	3.1	
(S_p)-Up(S)A	0.46	1.0	
(R_p)-Up(S)A	3.1	3.6	

[a] The assay contained in a 0.1–0.2-mL volume 40 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 1.0 mM UTP. For poly[d(A-T)] template the assay included 50 mM KCl, 1.0 mM initiator, 22 μg/mL enzyme, and 1.0 A_{260} unit/mL DNA; for T4 DNA template the assay included 50 mM KCl, 1.0 mM initiator, 9–17 μg/mL enzyme, and 1.8–3.6 nM DNA; for T7 DNA template the assay included 2.0 mM initiator, 29–57 μg/mL enzyme, and 5.2–9.2 nM DNA.

In order to determine which compounds were labeled in the pyrophosphate exchange reaction with $[^{32}P]PP_i$, we collected the charcoal-adsorbable radioactivity onto a Whatman GF/C disk as described above (except that the reaction was not quenched with 0.3 M EDTA since the EDTA was found to contain many UV-absorbing impurities). The radioactivity was then desorbed from the charcoal with a solution of 100:100:8 (v/v/v) water–ethanol–concentrated ammonia (Furth et al., 1962). The filtrate from this desorption step was concentrated by rotary evaporation to 0.1 mL. Aliquots (25 μL) of this solution were analyzed by LC, using buffer A (0.25 M KCl–0.05 M KH_2PO_4, pH 4.5) or buffer B as described above. Fractions (10–40 drops) from the effluent of the liquid chromatograph were collected manually and counted in 10 mL of water on the tritium channel of the liquid scintillation spectrometer. (The dead volume of the liquid chromatograph between the detector and the outlet was negligible.)

Results

Substrate Specificity for the Initiator. The initial rates for the formation of the first phosphodiester bond as a function of initiator and template are given in Table I (see Figure 1 for the structures of the diastereomers of ATPαS and Up(S)A mentioned in this table). All of the phosphorothioate analogues of ATP tested are substrates for the initiation step. This contrasts with the elongation step, where ATPαS, isomer B, is not a substrate (Eckstein et al., 1976; Armstrong et al., 1979); however, ATPαS, isomer A, is preferred over the B isomer in the initiation step by a factor of 3 (with T4 DNA as template) to 8 (with T7 DNA as template). ATPβS, isomer A, is preferred over the B isomer by a factor of 2 (with T7 DNA).

It is known that UpA can prime RNA synthesis on T4 DNA template (Downey et al., 1971). Of the four triphosphates (ATP, CTP, GTP, and UTP), we determined that only UTP

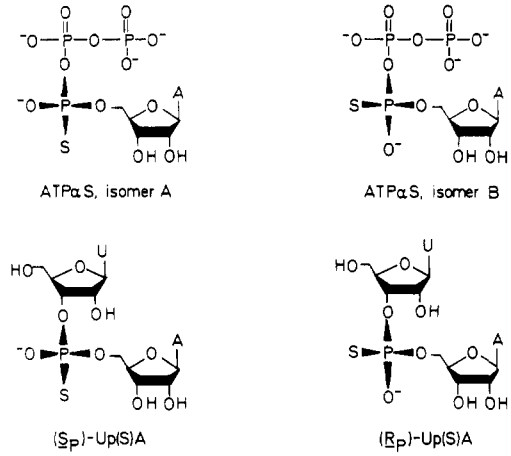

FIGURE 1: Absolute configuration of the isomers of ATPαS and Up(S)A.

Table II: Hydrolysis of Phosphorothioates by Snake Venom Phosphodiesterase[a]

time (h)	% hydrolysis pppAp-(S)U	CpAp-(S)U	(R_p)-Up(S)A	(S_p)-Up(S)A
1	44	70	72	0
4	44	95	100	0
20	78	100	100	0

[a] pppAp(S)U and CpAp(S)U were synthesized under initiation assay conditions as described in footnote *a* of Table I, using T7 DNA as template. The concentration of substrate in the hydrolysis reaction with snake venom phosphodiesterase was 10 A_{260} units/mL except for pppAp(S)U, which was 1 A_{260} unit/mL.

is able to add to this primer. Thus, the ability of the diastereomers of Up(S)A to substitute for UpA in the primed initiation reaction was followed by monitoring the formation of Up(S)ApU. Table I shows that the R_p isomer of Up(S)A is as good a substrate as UpA and that it is preferred over the S_p isomer by a factor of 4 (with T4 DNA) to 7 (with poly[d(A-T)]).

Stereochemical Course of the Initiation and Primed Initiation Reactions. T7 DNA has three major promoter sites for *E. coli* RNA polymerase, two of which initially synthesize pppApU and can be primed by CpA (Smagowicz & Scheit, 1978). By using T7 DNA as template, ATP or CpA as initiator, and UTPαS, isomer A, as substrate, we obtained the products pppAp(S)U and CpAp(S)U, respectively. (The B isomer of UTPαS is not a substrate for this reaction.) Table II shows the hydrolysis of these two products by snake venom phosphodiesterase compared to the hydrolysis of the two diastereomers of Up(S)A. It is known that snake venom phosphodiesterase hydrolyzes the R_p isomer of Up(S)A about 2000 times faster than the S_p isomer (Burgers & Eckstein, 1979). Since the products from both the initiation and primed initiation reactions are hydrolyzed at a rate comparable to that of (R_p)-Up(S)A [under the same conditions no hydrolysis of (S_p)-Up(S)A is observed], it is presumed that both products have the same absolute configuration as (R_p)-Up(S)A, viz., the R_p configuration. By analogy to ATPαS, isomer A (Burgers & Eckstein, 1978), the substrate, UTPαS, isomer A, has the S_p configuration; therefore, it follows that phosphodiester bond formation proceeds with inversion of configuration.

Pyrophosphate Exchange Reaction. Confirming and extending the results of Krakow & Fronk (1969), we found that ATP and the isomers of ATPαS and ATPβS catalyze the

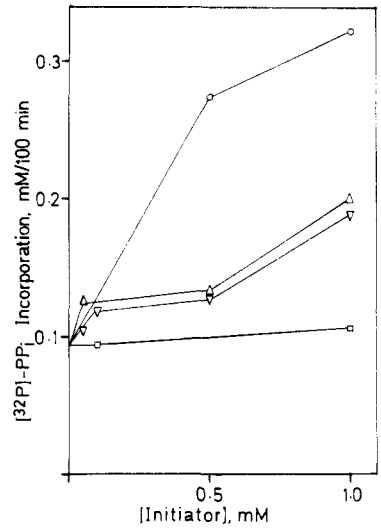

FIGURE 2: Pyrophosphate exchange as a function of initiator concentration. The assay included 1.0 mM UTP, 110 μg/mL RNA polymerase, 8.7 nM T7 DNA, and varying amounts of initiator. Other conditions are as described under Experimental Procedure. (○) ATP; (□) ADP; (▽) ATPαS, isomer A; (△) CpA.

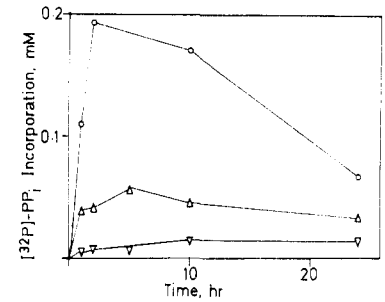

FIGURE 3: Pyrophosphate exchange with UTP analogues. The assay included 1.0 mM ATP, 1.0 mM UTP or an analogue, 88 μg/mL RNA polymerase, and 8.8 nM T7 DNA. Other conditions are as described under Experimental Procedure. (○) UTP; (▽) UTPαS, isomer A; (△) UTPαS, isomer B.

Table III: Distribution of Charcoal-Adsorbable Radioactivity from Pyrophosphate Exchange Reaction[a]

labeled species	substrate (%) UTP	UTPαS, A	UTPαS, B
PP_i	2	2	2
substrate	78	7[b]	0
ATP	19	89	75
unidentified	1	1	23

[a] The reaction assay included 1.0 mM ATP, 1.0 mM UTP or an analogue, 110 μg/mL RNA polymerase, and 8.7 nM T7 DNA. Other conditions are as described under Experimental Procedure. Incubation time was 100 min. [b] The label was found entirely in the A isomer of UTPαS.

exchange of pyrophosphate into UTP in the presence of RNA polymerase and poly[d(A-T)] as template (data not shown). In addition, ATP, ATPαS, isomer A, and CpA (but not ADP) catalyze pyrophosphate exchange into UTP in the presence of T7 DNA (see Figure 2); however, the optimal concentration of initiator is higher with the latter template than with poly[d(A-T)]. [The pyrophosphate exchange observed in the absence of initiator is probably caused by the presence of trace amounts of ATP in the UTP (Krakow & Fronk, 1969).]

When UTPαS is used in place of UTP in the pyrophosphate exchange reaction with ATP as initiator and T7 DNA as template, charcoal-adsorbable radioactivity is found with both A and B isomers (see Figure 3). Surprisingly, the B isomer,

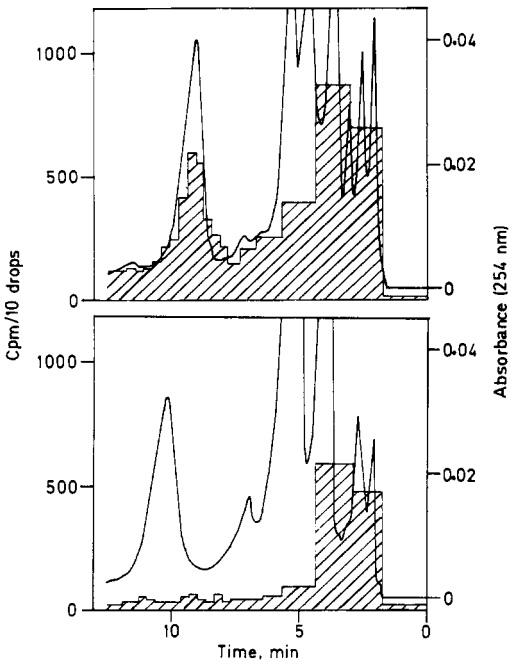

FIGURE 4: LC analysis of charcoal-absorbable radioactivity from pyrophosphate exchange reaction. The assay included 1.0 mM CpA, 1.0 mM UTPαS, 110 μg/mL RNA polymerase, and 8.7 nM T7 DNA. Other conditions are as described under Experimental Procedure. Incubation time was 100 min. The LC eluant was buffer A. The solid lines are the LC chromatograms. The hatched histograms represent the radioactivity. In the upper diagram UTPαS, isomer A, was the substrate; in the lower diagram UTPαS, isomer B, was the substrate. Elution times are 5.2 (CpA), 9.0 (UTPαS, A), and 10.2 (UTPαS, B) min. The radioactive peak eluting at ~3 min is PP_i.

which is not a substrate for the initiation reaction, shows more pyrophosphate incorporation than the A isomer; however, Table III shows that, with the isomers of UTPαS, there is no pyrophosphate exchange into the B isomer, and most of the pyrophosphate exchange is into ATP. This incorporation was found to be caused by ATP-catalyzed exchange into ATP at a minor promoter site which synthesized pppApA. To avoid this difficulty, we studied the CpA-catalyzed exchange into UTPαS with T7 DNA template. The results are shown in Figure 4. It can be clearly seen that pyrophosphate exchange into UTPαS, isomer A, yields the A isomer, whereas no pyrophosphate exchange occurs with the B isomer. (The elution times of the isomers of UTPαS under the conditions used were 9.0 and 10.2 min for isomers A and B, respectively.) Qualitatively identical results were obtained by using ATP as initiator, although the results were more difficult to interpret for the reason explained above. Hence, the pyrophosphate exchange reaction occurs with retention of configuration.

Discussion

Previous studies [see Krakow et al. (1976) for a review] have shown that the postulated initiation nucleotide site has a greater substrate specificity than the elongation nucleotide site with respect to the base and sugar moieties but that the reverse is true with respect to the triphosphate moiety. The present results confirm the latter point since it is found that the B isomer of ATPαS will function as an initiating substrate but not as an elongating substrate (Eckstein et al., 1976). One surprising aspect of the initator specificity is the finding that the A (i.e., S_p) isomer of ATPαS (Burgers & Eckstein, 1979) is a better initiator than the B (i.e., R_p) isomer whereas the R_p isomer of Up(S)A is a better initiator than (S_p)-Up(S)A, even though the S_p isomers of both ATPαS and Up(S)A have

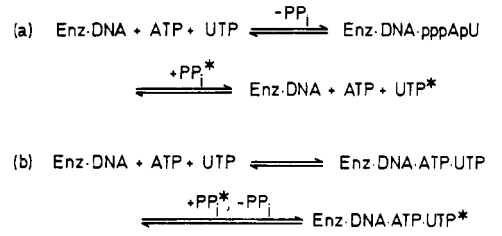

FIGURE 5: Possible mechanisms for the pyrophosphate exchange reaction.

the same absolute configuration (see Figure 1). One possible explanation is the existence of separate subsites in the initiation site for the triphosphate and the growing RNA chain. Another possibility is the existence of different enzyme conformations which depend upon whether the initiation site is occupied by the initiating triphosphate or by the growing RNA chain. A third explanation involves the initial binding of the initiator to the elongation site followed by translocation into the initiation site. (Of course, these explanations are not mutually exclusive.) Whatever the explanation, the results obtained with the phosphorothioate analogues show that one should be careful about using primed initiation reactions as models for the initiation step.

By determining the absolute configuration of the product formed from UTPαS, isomer A, and ATP or CpA with T7 DNA as template, it was possible to conclude that both the initiation and primed initiation reactions proceed with inversion of configuration at the α-phosphorus of the substrate. This result is identical with the stereochemistry of the elongation step of RNA polymerase action (Eckstein et al., 1976; Burgers & Eckstein, 1978). It may therefore be concluded that all the phosphodiester bond-forming reactions catalyzed by RNA polymerase proceed by the same mechanism, viz., an S_N2 attack on the α-phosphorus of the elongating nucleotide by the 3′-OH group of the growing RNA chain.

The pyrophosphate exchange reaction has usually been assumed to be the reverse of the phosphodiester bond-forming step (Krakow & Fronk, 1969); however, alternative theories were viable, e.g., the pyrophosphorolysis of enzyme-bound substrate (see Figure 5). In the former case, retention of configuration would be expected at the α-phosphorus; in the latter case, inversion or racemization of configuration would be expected. By determining the absolute configuration of pyrophosphate-exchanged UTPαS, it is possible to distinguish between the two alternatives. Since retention of configuration was observed in the pyrophosphate exchange reaction, the latter possibility is excluded, and all the results are consistent with pyrophosphate exchange being the reverse of the phosphodiester bond-forming reaction.

Acknowledgments

The authors are indebted to Drs. P. M. J. Burgers and H. Wiedner for valuable advice and discussions and to G. Witzel for her excellent technical assistance.

References

Abelson, J., & Thomas, C. A., Jr. (1966) *J. Mol. Biol. 18*, 262–288.

Armstrong, V. W., Yee, D., & Eckstein, F. (1979) *Biochemistry* (following paper in this issue).

Burgers, P. M. J., & Eckstein, F. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4798–4800.

Burgers, P. M. J., & Eckstein, F. (1979) *Biochemistry 18*, 592–596.

Chamberlin, M. J. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M. J., Eds.) pp 17–67, Cold Spring Harbor

Laboratory, Cold Spring Harbor, NY.
Downey, K. M., Jurmark, B. S., & So, A. G. (1971) *Biochemistry 10*, 4970–4975.
Eckstein, F. (1975) *Angew. Chem. 87*, 179–186 (*Angew. Chem., Int. Ed. Engl. 14*, 160–166).
Eckstein, F. (1979) *Acc. Chem. Res. 12*, 204–210.
Eckstein, F., & Goody, R. S. (1976) *Biochemistry 15*, 1685–1691.
Eckstein, F., Armstrong, V. W., & Sternbach, H. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2987–2990.
Freifelder, D. (1970) *J. Mol. Biol. 54*, 567–577.
Furth, J. J., Hurwitz, J., & Anders, M. (1962) *J. Biol. Chem. 237*, 2611–2619.
Jaffe, E. K., & Cohn, M. (1978) *J. Biol. Chem. 253*, 4823–4825.
Krakow, J. S., & Fronk, E. (1969) *J. Biol. Chem. 244*, 5988–5993.
Krakow, J. S., Rhodes, G., & Jovin, T. M. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M. J., Eds.) pp 127–157, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Midelfort, C. F., & Sarton-Miller, I. (1978) *J. Biol. Chem. 253*, 7127–7229.
Smagowicz, W. J., & Scheit, K. H. (1978) *Nucleic Acids Res. 5*, 1919–1932.
So, A. G., & Downey, K. M. (1970) *Biochemistry 9*, 4788–4793.
Stahl, S. J., Schlimme, E., & Eckstein, F. (1974) *FEBS Lett. 40*, 241–246.
Sternbach, H., Engelhardt, R., & Lezius, A. G. (1975) *Eur. J. Biochem. 60*, 51–55.
Thomas, C. A., Jr., & Abelson, J. (1966) in *Procedures in Nucleic Acid Research* (Cantoni, G. L., & Davies, D. R., Eds.) pp 553–561, Harper and Row, New York.

Mechanistic Studies on Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerase from *Escherichia coli* Using Phosphorothioate Analogues. 2. The Elongation Reaction†

Victor William Armstrong, David Yee, and Fritz Eckstein*

ABSTRACT: The metal ions Mn(II), Co(II), Zn(II), and Cd(II) are able to replace Mg(II) in the transcription of poly[d(A-T)] by deoxyribonucleic acid (DNA) dependent ribonucleic acid (RNA) polymerase from *Escherichia coli*. In the presence of Mg(II), the A isomers of adenosine 5′-*O*-(1-thiotriphosphate) (ATPαS) and uridine 5′-*O*-(1-thiotriphosphate) (UTPαS) are substrates for RNA polymerase (K_m = 0.2–0.4 mM) whereas the B isomers are weak inhibitors ($K_i \simeq 2$ mM). Replacement of Mg(II) by Co(II) or Cd(II) does not alter the substrate properties of these analogues, the A isomers remaining substrates while the B isomers are inactive. Both diastereomers of adenosine 5′-*O*-(2-thiotriphosphate) (ATPβS) are substrates with Mg(II), having similar K_m values [0.3–0.4 mM, K_m(MgATP) = 0.05 mM]. However, with Cd(II) only the B isomer is a substrate [K_m = 0.8 mM, K_m(CdATP) = 1.2 mM], the A isomer being a weak inhibitor ($K_i \geq 5$ mM). Since Cd(II) chelates mainly to the sulfur atom of phosphorothioates and Mg(II) to the oxygen atom [Jaffe, E. K., & Cohn, M. (1978) *J. Biol. Chem. 253*, 4823–4825], a model is proposed on the basis of these data for the binding of the metal–ATP complex to the elongation site of RNA polymerase. Thus, the Δ isomer of the bidentate MgATP complex [Cornelius, R. D., & Cleland, W. W. (1978) *Biochemistry 17*, 3279–3286] is the active form for RNA polymerase, and there is an additional binding of the nonchelated β-oxygen to the enzyme. The metal atom is not bound to the α-phosphate, but there is a stereospecific interaction between a positive group on the enzyme and one of the α-phosphate atoms.

The syntheses and separation of the diastereomers of ATPαS[1] and ATPβS (Eckstein & Goody, 1976) have enabled their use in the study of enzyme mechanisms (Eckstein, 1975, 1979). The asymmetry resulting from the introduction of the phosphorothioate group can be utilized to investigate not only the stereochemistry of phosphodiester bond formation [for reviews, see Eckstein (1979) and Westheimer (1979)] but also the geometry of the substrate binding site by comparing the substrate properties of the two diastereomers. Furthermore, information as to the active form of the divalent metal ion–ATP complex can be derived by the use of metal ions differing in their affinities for the oxygen and sulfur atoms of the phosphorothioate groups. Thus, on the basis of nuclear magnetic resonance studies, Jaffe & Cohn (1978) concluded that Mg(II) chelates preferentially to the oxygen atom of the β-phosphorus of ATPβS but that Cd(II) chelates to the sulfur atom. In the presence of Mg(II), the B isomer of ATPβS is the preferred substrate for hexokinase, the A isomer being inactive, whereas with Cd(II) the reverse is true, the A isomer being a substrate and the B isomer inactive. In the case of Co(II), both isomers are substrates.

The stereochemistry of phosphodiester bond formation has been determined for the elongation reaction (Eckstein et al., 1976; Burgers & Eckstein, 1978) and for the initiation and pyrophosphate exchange reactions (Yee et al., 1979) of DNA-dependent RNA polymerase from *Escherichia coli*. This enzyme requires a divalent metal ion such as Mg(II) for its polymerizing activity, and nuclear magnetic resonance studies with Mn(II) in place of Mg(II) (Koren & Mildvan, 1977) have indicated that there is one tight binding site for this ion on the enzyme. In this investigation we report the stereospecificity of incorporation of phosphorothioate substrate

† From the Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, D-3400 Göttingen, Federal Republic of Germany. *Received February 22, 1979.* This research was supported in part by a grant from the Deutsche Forschungsgemeinschaft.

[1] The abbreviations used are as in the previous paper (Yee et al., 1979).

0006-2960/79/0418-4120$01.00/0