Krimsky, I., & Racker, E. (1963) *Biochemistry 2*, 512–518.
MacQuarrie, R. A., & Bernhard, S. A. (1971) *Biochemistry 10*, 2456.
Malhotra, O. P., & Bernhard, S. A. (1968) *J. Biol. Chem. 243*, 1243–1252.
Malhotra, O. P., & Bernard, S. A. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2077–2081.
Malhotra, O. P., Srinivasan, & Srivastava, D. K. (1978) *Biochim. Biophys. Acta 526*, 1–12.
Monod, J., Wyman, J., & Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88–118.
Perham, R. N., & Harris, J. I. (1963) *J. Mol. Biol. 7*, 316–320.
Racker, E., & Krimsky, I. (1952) *J. Biol. Chem. 198*, 731–743.
Scheek, R. M., & Slater, E. C. (1978) *Biochim. Biophys. Acta 526*, 13–24.
Schwendimann, B., Ingbar, D., & Bernhard, S. A. (1976) *J. Mol. Biol. 108*, 123–138.
Seydoux, F., & Bernhard, S. A. (1974) *Biophys. Chem. 1*, 161–174.
Seydoux, F., Bernhard, S. A., Pfenninger, O., Payne, M., & Malhotra, O. P. (1973) *Biochemistry 12*, 4290–4300.
Seydoux, F., Keleman, N., Kellershohn, N., & Roucous, C. (1976) *Eur. J. Biochem. 64*, 481.
Stadtman, E. R. (1970) *Enzymes, 3rd Ed. 1*, 397–459.

Properties of P^3 Esters of Nucleoside Triphosphates as Substrates for RNA Polymerase from *Escherichia coli*†

Wieslaw J. Smagowicz,‡ José V. Castell,§ Robert M. Clegg, and Karl-Heinz Scheit*

ABSTRACT: P^3-[(2,4-Dinitrophenyl)amino]ethyl (DNPNHEt) and P^3-methyl phosphate esters of nucleoside 5′-triphosphates have been synthesized. Their properties as substrates in the initiation and elongation steps of transcription have been examined by using RNA polymerase from *Escherichia coli* and poly[d(A-T)] or T7 DNA as templates. It is shown that transcription can be initiated by ATP-EtNHDNP and that 2,4-dinitrophenyl residues are incorporated at the 5′ end of the RNA molecules. Steady-state kinetic experiments of abortive initation on promoters A1 and A3 of T7 DNA revealed that ATP-EtNHDNP, ADP-EtNHDNP, and ATP-OCH_3 have lower K_m values and markedly reduced V_{max} values compared to those of ATP. The two classes of esters, NTP-EtNHDNP and NTP-OCH_3, were found to differ regarding their utilization as substrates for elongation. Both ATP-OCH_3 and UTP-OCH_3 are substrates for transcription. However, only the pyrimidine derivatives of NTP-EtNHDNP are elongation substrates which release DNPNHEt-PP upon utilization. This dramatic difference between the purine and pyrimidine derivatives of NTP-EtNHDNP reflects a selective process in the transcriptional complex for purines and pyrimidines.

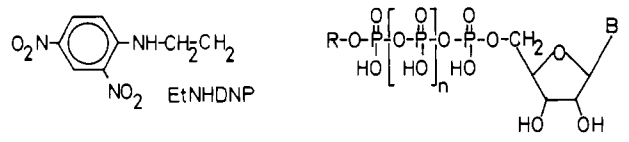
Several methods for introducing labels into RNA have been developed in the past in order to provide the newly synthesized RNA with a spectroscopic or functional marker. Nucleotide analogues which can be incorporated into RNA polymers by RNA polymerase are useful probes of the structure and function of the RNA itself and also provide information about the requirements and mechanism of transcription (Chamberlin, 1974). Most of the analogues have been selected to study the substrate requirements of the enzymatic process for the chemical structure of the nucleotide base. Base analogues which have useful spectral characteristics include those that are fluorecent, such as formycin 5′-triphosphate (Darlix et al., 1971), n^2ATP and n^2h^6ATP (Ward et al., 1969; Rackwitz & Scheit, 1977), and 7-deazanebularine (Ward & Reich, 1972). s^4UTP (Cramer et al., 1971) and s^6GTP (Darlix et al., 1973) both have absorption maxima above 300 nm which can be easily recognized. s^2UTP, 5′-mecuripyridine (Livingstone et al., 1976), and s^4UTP (Rackwitz & Scheit, 1977) can interact with solid supports via sulfur–mecury bonds and are therefore easily recognizable. Although the usefulness of the latter analogues for separating RNA transcripts has been well demonstrated in the above studies, other functional groups on nucleotides which can be incorporated into RNA are needed, especially since the above sulfur analogues are unstable due to the functional group (van Broeckhoven & de Wachter, 1978).

Another method of labeling RNA takes advantage of the initiation step of RNA synthesis (Chamberlin, 1976). This initiation reaction involves the phosphorylation of the 3′-ribofuranosyl position of a purine nucleoside 5′-triphosphate, ATP or GTP, by the 5′-α-phosphate of the next substrate, and therefore the purines act as primers of RNA synthesis. The 5′-triphosphate residues of the primers constitute the 5′ end of the newly synthesized RNA chain, and it should be feasible to link substituents to the γ-phosphate of these primers without significantly impairing their primer function.

Such derivatives of ATP have been known for some time. Grachev & Zaychikov (1974) showed that ATP-P^3-anilidate functions efficiently as a substitute for ATP in elongation, and although not explicitly shown, it is anticipated that this analogue participates in initiation. Armstrong & Eckstein (1976) investigated the properties ATP P_3 esters and fluoro esters as substrates for RNA polymerase from *Escherichia coli* and observed that those analogues functioned either as substrates with unfavorable kinetic parameters or as inhibitors. They did not investigate if such analogues function as primers in

† From the Abteilung Molekulare Biologie, Max-Planck-Institut für Biophysikalische Chemie, D-34 Göttingen, West Germany. *Received October 16, 1980; revised manuscript received March 16, 1981.* J.V.C. gratefully acknowledges the receipt of an EMBO long-term fellowship.
‡ Present address: Institute of Biophysics and Biochemistry, Polish Academy of Sciences, Warsaw, Poland.
§ Present address: Centro de Investigación, Department of Enzimologia, Ciudad Sanitaria La Fe, Valencia, Spain.

0006-2960/81/0420-5538$01.25/0



	R	B	n
ADP-EtNHDNP	EtNHDNP	ADENINE	0
ATP-EtNHDNP	EtNHDNP	ADENINE	1
GTP-EtNHDNP	EtNHDNP	GUANINE	1
UTP-EtNHDNP	EtNHDNP	URACIL	1
CTP-EtNHDNP	EtNHDNP	CYTOSINE	1
ATP-OCH_3	CH_3	ADENINE	1
UTP-OCH_3	CH_3	URACIL	1

FIGURE 1: Structural formulas of nucleotide analogues.

the initiation of transcription. Recently Yarbrough et al. (1979) reported the excellent substrate properties of adenosine 5′-triphosphate P^3-[5-(hydroxysulfonyl)naphthyl-1-amidate] for *E. coli* RNA polymerase.

We have prepared [(2,4-dinitrophenyl)amino]ethyl esters of nucleoside 5′-diphosphates and nucleoside 5′-triphosphates and explored their behavior in initiation and elongation of transcription. The 2,4-dinitrophenyl derivatives were chosen because of their potential interaction with 2,4-dinitrophenyl-specific antibodies. Similar experiments are also presented for the esters ATP-OCH_3 and UTP-OCH_3[1]. The properties of the nucleotide derivatives presented in this study (Figure 1) are compared to those of similar analogues.

Materials and Methods

Enzymes. RNA polymerase holoenzyme from *E. coli* was purified according to Zillig et al. (1970) through the DEAE-cellulose step and then by affinity chromatography on heparin-Sepharose as described by Sternbach et al. (1975). A unit of activity is defined to be a nanomole of total nucleotide incorporated per hour, calculated from the linear portion of a kinetic curve. The isolated holoenzyme had a specific activity of 7000 units/mg of protein assayed with T7 DNA by rifampicin challenge, at 37 °C (Mangel & Chamberlin, 1974). Alkaline phosphatase (EC 3.1.3.1) and snake venom phosphodiesterase (EC 3.1.4.1) were purchased from Boehringer (Mannheim, West Germany).

Chemicals. T7 DNA was isolated from purified phage as described in the literature (Thomas & Abelson, 1966). The following chemicals were obtained from the indicated sources: [^{14}C]UTP (specific activity 486 mCi/mmol), [^{14}C]CTP (specific activity 500 mCi/mmol), and [^{14}C]ATP (specific activity 502 mCi/mmol) from Amersham-Buchler; poly[d-(A-T)], ATP, GTP, UTP, CTP, and CpA from Boehringer; ADP, GDP, CDP, and UDP from Pharma Waldorf GmbH (Duesseldorf, West Germany); morpholine, dicyclohexylcarbodiimide (DCCI), diphenylphosphoryl chloride, and $POCl_3$ from Merck (Darmstadt, West Germany). All other chemicals were analytical grade.

Assay of RNA Polymerase. Buffer A (40 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM mercaptoethanol) was used throughout. The temperature for the assays was 37 °C. The assay mixture contained buffer A, template, enzyme, and substrates in concentrations as specified in tables or figure legends. The progress of reaction was followed either by chromatography on paper strips (Scheit & Faerber, 1975) or by precipitation of synthesized RNA with ice-cold 3.5% perchloric acid containing 50 mM pyrophosphate and collection of the precipitated polynucleotides on Whatman GF/C glass-fiber filters.

Transcription of Poly[d(A-T)] in the Presence of UTP-EtNHDNP and ATP. The 0.1-mL mixture contained buffer A, 0.3 mM UTP-EtNHDNP, 0.25 mM ATP, [^{14}C]ATP (0.75 Ci), 0.3 mM poly[d(A-T)], and 9.5 μg of enzyme. Incubation was carried out for 15 h at 37 °C. A 5-μL aliquot of the mixture was subjected to paper-strip chromatography. The yield of polymerization was 22%. The remaining reaction mixture was chromatographed on PEI–cellulose (thin layer) with 1 M NaCl. The chromatography revealed the presence of two yellow substances in the reaction mixture with R_f 1.22 and R_f 0.44 relative to the [(2,4-dinitrophenyl)amino]ethyl phosphate. The substances were eluted from PEI–cellulose with 2 M triethylammonium bicarbonate. After evaporation to dryness, the residues were dissolved in 5 mM Tris-HCl, pH 7. On the basis of their absorption spectra and the sensitivity to alkaline phosphatase, the compound with R_f 1.22 was UTP-EtNHDNP, and the compound with R_f 0.44 was [(2,4-dinitrophenyl)amino]ethyl pyrophosphate.

Hydrolysis of EtNHDNP Derivatives by Phosphodiesterase or Alkaline Phosphatase. The respective EtNHDNP derivatives (0.1 μmol) in 50 μL of Tris-HCl, pH 7 (10 mM), were incubated with either 5 μg of phosphodiesterase or 5 μg of alkaline phosphatase for 1.5 h at 37 °C. The reaction mixture was subjected to PEI–cellulose thin-layer chromatography.

Synthesis of [(2,4-Dinitrophenyl)amino]ethyl Pyrophosphate. Phosphoric acid (150 μmol) and tri-*n*-butylamine (0.11 mL, 0.463 mmol) were dissolved in a mixture of pyridine and water. The solution was taken to dryness, the residue was rendered anhydrous by repeated coevaporations with anhydrous dimethylformamide, and then [(2,4-dinitrophenyl)-amino]ethyl phosphormorpholidate (15 μmol) was added. The reaction mixture was kept in the dark for 24 h and then added dropwise to 10 mL of 1% $NaClO_4$ in acetone. The precipitated sodium salts were collected by centrifugation. Analysis by PEI–cellulose thin-layer chromatography of the crude mixture in 1 M NaCl showed the presence of two yellow substances with R_f 3.06 and 0.44, respectively [relative to [(2,4-dinitrophenyl)amino]ethyl phosphate]. On the basis of the mobility as well as on the fact that it was hydrolyzed by alkaline phosphatase, the material with R_f 0.44 was [(2,4-dinitrophenyl)amino]ethyl pyrophosphate.

Synthesis of [(2,4-Dinitrophenyl)amino]ethyl Phosphate. $POCl_3$ (4.5 mL, 50 mmol) was added to a solution of [(2,4-dinitrophenyl)amino]ethanol (2.3 g, 10 mmol) in 10 mL of triethyl phosphate. The reaction mixture was kept at 0 °C for 3 h. The mixture was then poured into 200 mL of ice–water containing 10 mL of pyridine. The resulting solution was extracted with chloroform. A precipitate was filtered off and the filtrate adjusted to pH 11 with 1 M LiOH. The precipitated Li_3PO_4 was removed by filtration and the filtrate evaporated to dryness. The residue was extracted with ace-

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DNPNHEt-P (or EtNHDNP), [(2,4-dinitrophenyl)amino]ethyl phosphate; DNPNHEt-PP, [(2,4-dinitrophenyl)amino]ethyl pyrophosphate; UTP-EtNHDNP, P^3-[(2,4-dinitrophenyl)amino]ethyl P^1-(5′-uridine) triphosphate; ATP-EtNHDNP, P^3-[(2,4-dinitrophenyl)amino]ethyl P^1-(5′-adenosine) triphosphate; ADP-EtNHDNP, P^2-[(2,4-dinitrophenyl)amino]ethyl P^1-(5′-adenosine) diphosphate; GTP-EtNHDNP, P^3-[(2,4-dinitrophenyl)amino]ethyl P^1-(5′-guanosine) triphosphate; CTP-EtNHDNP, P^3-[(2,4-dinitrophenyl)-amino]ethyl P^1-(5′-cytidine) triphosphate; DNPNHEt-pppApU, P^3-[(2,4-dinitrophenyl)amino]ethyl uridylyl-(5′–3′)-adenosine 5′-triphosphate; ATP-OCH_3 P^3-methyl P^1-(5′-uridine)triphosphate; ATP-OCH_3, P^3-methyl P^1-(5′-adenosine) triphosphate. The P^n notation is used throughout. α, β, and γ are used only when directly referring to the position along the polyphosphate chain.

tone–ethanol (1:1 v/v), dissolved in water, and applied to a DEAE-Sephadex A-25 column (5 × 20 mL). The column was eluted with a 6-L linear gradient of triethylammonium bicarbonate from 0 to 0.3 M. The fractions were analyzed by thin-layer chromatography on PEI–cellulose employing 0.25 M NaCl. Appropriate fractions were pooled and evaporated to dryness. The residual gum crystallized as the triethylammonium salt upon treatment with ether. The yield was 2.01 g (47%): UV (water, pH 7) 360 nm (ϵ = 16 500 M^{-1} cm^{-1}) and 260 (ϵ = 8600 M^{-1} cm^{-1}). Anal. Calcd. for $C_{14}H_{25}O_8N_4P \cdot H_2O$ (mol wt 426.4): C, 39.4; H, 6.4; N, 13.1; P, 7.3. Found C, 39.4; H, 6.1; N, 13; P, 8.9.

Synthesis of [(2,4-Dinitrophenyl)amino]ethyl Phosphormorpholidate. The preparation followed closely that of Moffatt & Khorana (1961). After completion of the reaction, dicyclohexylurea was removed by filtration. The filtrate was evaporated to a small volume, diluted with water, and extracted with ether. The aqueous phase was evaporated, and the residue was dried by repeated codistillation with anhydrous dimethylformamide and used in reactions without further processing. The material was homogeneous as shown by silica gel thin-layer chromatography in the 7:1:2 solvent[2] [[(2,4-dinitrophenyl)amino]ethyl phosphate, R_f 1; morpholidate, R_f 1.83].

Synthesis of P^3-[(2,4-Dinitrophenyl)amino]ethyl P^1-(5′-Nucleoside) Triphosphates. Method A. The triethylammonium salts of nucleoside 5′-triphosphates were prepared in the following manner. The sodium salt (1 mmol) was dissolved in 10 mL of water and the solution applied to a DEAE-Sephadex A-25 (HCO_3^- form, 3 × 3 cm) column. The column was washed with 100 mL of water. Elution of the nucleotide was carried out with 1 M triethylammonium bicarbonate. The eluate was concentrated to a gum by flash evaporation. The residue was repeatedly codistilled with methanol until traces of triethylamine were removed. The nucleoside 5′-triphosphate triethylammonium salt (0.1 mmol) was dissolved in 1 mL of methanol, and 0.3 mmol of tri-*n*-butylamine was added. The solution was concentrated to a gummy residue, which was dried by coevaporation with anhydrous pyridine at 0.1 torr and 25 °C. To the remainder, which was dissolved in 1 mL of anhydrous dimethylformamide, was added diphenylphosphoryl chloride (0.1 mmol, 0.035 mL); this was immediately followed by tri-*n*-butylamine (0.1 mmol, 0.024 mL). The mixture was vigorously agitated for approximately 1 min to dissolve an eventual precipitate and then kept for 30 min at room temperature. After addition of 2-[(2,4-dinitrophenyl)amino]ethanol (195 mg, 1 mmol), the reaction was allowed to proceed in the dark. The yellow solution was then added dropwise with stirring to 20 mL of a 1% solution of $NaClO_4$ in acetone. The precipitated sodium salts were collected by centrifugation, resuspended in acetone, and centrifuged again. The yellow precipitate was dissolved in the minimum amount of water, and the solution was applied to one sheet of Whatman 3MM chromatography paper (50 × 70 cm). Descending paper chromatography was carried out with 7:1:2 solvent. The slowest moving yellow band corresponded to the P^3-[(2,4-dinitrophenyl)amino]ethyl derivative. Elution of the nucleotide from the paper was carried out with water. The yield, as determined spectrophotometrically from the absorbance at 360 nm, was usually on the order of 15%. A faster moving yellow band, which was identified as P^2-[(2,4-dinitrophenyl)amino]ethyl P^1-(5′-nucleoside) diphosphate, was generally found as a byproduct of the reaction. The yield of this species was on the order of 10%.

Method B. The nucleoside 5′-diphosphate bis(tri-*n*-butylammonium) salt (0.2 mmol) was dried by repeated coevaporations with anhydrous pyridine. The residual gum was dissolved in 2 mL of anhydrous dimethyl sulfoxide. After addition of [(2,4-dinitrophenyl)amino]ethyl phosphormorpholidate (0.05 mmol) in 0.5 mL of dimethyl sulfoxide, the reaction mixture was kept for 3 days at room temperature in the dark. The reaction mixture was added dropwise with stirring to 20 mL of 1% $NaClO_4$ in acetone. The precipitate was collected by centrifugation and dissolved in a minimal amount of water, and the solution was subjected to preparative descending paper chromatography on Whatman 3MM paper employing the 7:1:2 solvent. The P^3-[(2,4-dinitrophenyl)-amino]ethyl derivative corresponded to the slowest moving yellow band. The desired material was isolated by elution with water in yields ranging from 40% to 50%.

The synthesis of P^2-[(2,4-dinitrophenyl)amino]ethyl P^1-5′-ADP was carried out with a similar procedure starting from AMP tri-*n*-butylammonium salt.

Synthesis of P^3-Methyl P^1-(5′-Nucleoside) Triphosphates. Nucleoside 5′-triphosphate bis(tri-*n*-butylammonium) salts (0.1 mmol), prepared as described above, were rendered anhydrous by repeated coevaporations with anhydrous pyridine. The residue was dissolved in 1 mL of dimethylformamide (anhydrous) and diphenylphosphoryl chloride (0.1 mmol, 0.035 mL) followed by tri-*n*-butylamine (0.1 mmol, 0.045 mL). After vigorous agitation for 1 min, the mixture was kept for 30 min at room temperature. Anhydrous methanol (1 mL) was added and the mixture kept for 15 h at room temperature. The reaction mixture was concentrated by flash evaporated at 0.1 torr to a small volume and subjected to preparative descending paper chromatography on Whatman 3MM paper in 7:1:2 solvent. The desired compound moved slightly faster than UDP and was eluted form the paper with water. The yield as determined spectrophotometrically ranged from 40% to 50%. The P^3-methyl esters were characterized by hydrolysis with phosphodiesterase and phosphate analysis and as expected were completely resistant to alkaline phosphatase.

Preparative Synthesis and Structural Proof of DNPNHEt-pppApU. A 2.01-mL sample of the reaction mixture contained 0.15 mM poly[d(A-T)], 80 μg of enzyme, 0.275 mM ATP-EtNHDNP, and 0.385 mM [^{14}C]UTP (specific activity 43 × 10^6 cpm/ nmol) in buffer A. The mixture was incubated for 3 h at 37 °C and then subjected to preparative descending paper chromatography (Schleicher & Schüll 2043b, acid washed) and eluted with 55:10:35 solvent. Two yellow bands with R_f values of 1.43 and 0.92 relative to UTP were separated. The band of R_f 0.92 was eluted with water. The eluate was concentrated and rechromatographed under the same conditions. Finally 2.06 A_{360} units was obtained with a total radioactivity of 1.01 × 10^6 cpm. When an extinction coefficient of 16 900 M^{-1} cm^{-1} (360 nm) and the known specific activity of [^{14}C]UTP was used, the ratio 0.024 μmol of DNP:0.023 μmol of UMP in the isolated material was calculated. The measured ratio A_{360}/A_{260} from the absorption spectrum was 0.47. With the extinction coefficients 16 900 M^{-1} cm^{-1} (360 nm) for the DNP chromophore and 10 000 M^{-1} cm^{-1} (260 nm) for uracil and 15 400 M^{-1} cm^{-1} (260 nm) for adenine, the ratio A_{360}/A_{260} = 0.49 was calculated. The isolated product was stable against alkaline phosphatase.

Results

Synthesis of Nucleotide [(2,4-Dinitrophenyl)amino]ethyl Esters. The synthesis of the nucleotide esters involved acti-

[2] The 7:1:2 solvent is 2-propanol–concentrated NH_4OH–H_2O (7:1:2 v/v), and the 55:10:35 solvent is 2-propanol–concentrated NH_4OH–H_2O (55:10:35 v/v).

Table I: Ultraviolet Absorption Spectra

compd	absorption maxima and minima (nm)	ratio DNP:base[a]	ratio DNP:P[b]
DNPNHEtOH	λ_{max} 360 (16 930), λ_{max} 265 (9010), λ_{min} 255 (8020), λ_{min} 260 (8680), λ_{min} 270 (8160)[c]		
DNPNHEt-P	λ_{max} 360 (16 700), λ_{max} 265 (8700)		
UTP-EtNHDNP	λ_{max} 360 (16 900), λ_{max} 260 (18 680)	1:1.023	1:3.1
CTP-EtNHDNP	λ_{max} 360 (16 070), λ_{max} 265 (15 700)	1:1.04[d]	1:2.98
ATP-EtNHDNP	λ_{max} 360 (16 900), λ_{max} 260 (24 080)	1:1.07	1:3.37
ADP-EtNHDNP	λ_{max} 360 (16 900), λ_{max} 260 (24 080)	1:0.91	1:2.02
GTP-EtNHDNP	λ_{max} 360 (16 900), λ_{max} 254 (21 700)	1:0.89	1:3.1
DNPNHEt-pppApU	λ_{max} 360 (17 600), λ_{max} 260 (37 600),[e] 425 (sh)	1:1.04[f]	

[a] Calculated from the absorption spectrum neglecting hyperchromicity. [b] Phosphate was determined according to Chen et al. (1956). [c] Numbers given in parentheses are molar extinction coefficients (mol^{-1} cm^{-1}). [d] Calculated from the absorption spectrum after hydrolysis by phosphodiesterase. [e] Concentration determined from known specific activity of [^{14}C]UMP. [f] Calculated from absorption at 360 nm and known specific activity of [^{14}C]UMP.

Table II: R_f Values of DNPNHEt Derivatives in PEI-Cellulose Thin-Layer Chromatography

compd	R_f value[a] 1 M NaCl	R_f value[a] 0.25 M NaCl
p-EtNHDNP	1.0	1.0
pp-EtNHDNP	0.44	0.095
ATP	0.35	
CDP	0.95	
AMP	1.12	1.62
CMP	1.54	
DNPNHEt-phosphormorpholidate	3.06	
UTP-EtNHDNP	1.22	
CTP-EtNHDNP	1.54	
ATP-EtNHDNP	1.13	
ADP-EtNHDNP	1.16	1.62

[a] All R_f values are normalized with respect to the mobility of p-EtNHDNP.

Table III: Transcription of T7 DNA in the Presence of UTP-EtNHDNP and CTP-EtNHDNP[a]

substrate	K_m (μM)	V_{max}^{app} (pmol/min)
UTP-EtNHDNP	330	6.8
CTP-EtNHDNP	170	4.8
UTP	16	260

[a] The incubation mixture (0.1 mL) contained 40 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM mercaptoethanol, 0.165 pmol of T7 DNA, 3.8 μg of enzyme, 0.15 mM CpA, and either GTP, ATP, and UTP or GTP, ATP, and CTP at 5 μM. [^{14}C]UTP (3×10^5 cpm) or [^{14}C]CTP (3×10^5 cpm) was employed as radioactive substrate. The concentrations of UTP-EtNHDNP or CTP-EtNHDNP, respectively, have been varied from 5 to 250 μM. V_{max}^{app} is the maximal velocity of the transcription at substrate saturation under the experimental conditions of DNA and enzyme concentration employed.

vation of the corresponding nucleotide by reaction with diphenylphosphoryl chloride followed by alcoholysis of the intermediary anhydride (Michelson, 1964). Isolation of the desired reaction products could not be achieved by DEAE-anion-exchange chromatography due to the abnormally high affinity of the DNPNHEt derivatives to anion-exchange supports. Preparative paper chromatography was found to be a convenient method for isolating the DNPNHEt esters. The P^3-methyl esters of ATP and UTP were prepared in a similar manner. The yields of these condensation reactions varied from 15% to 25%. The ratio of base to DNP in the DNPNHEt derivatives could be derived from the absorption spectra due to the strong absorption of the DNP chromophore at 360 nm. The results are listed in Table I. CTP-EtNHDNP is the only derivative which displays a substantial hyperchromicity in the absorption spectrum. However, after hydrolysis with phosphodiesterase, the ratio DNP/cytosine which was calculated from the absorption spectrum of the hydrolysis mixture agreed with expectations. All P^2 and P^3 esters are stable against hydrolysis by alkaline phosphatase, but they are all hydrolyzed by phosphodiesterase from snake venom. Hydrolysis was conveniently monitored by PEI–cellulose thin-layer chromatography. The relative mobilities of the DNPNHEt derivatives and the hydrolysis products are summarized in Table II. Hydrolysis of P^3-EtNHDNP derivatives of nucleoside 5′-triphosphates by phosphodiesterase yielded DNPNHEt-PP and the corresponding nucleoside 5′-monophosphate. These findings are in agreement with observations made by Yarbrough et al. (1979). The stability of the prepared esters is comparable to those of the respective nucleoside 5′-polyphosphates.

Substrate Properties of P^3 Esters of Nucleoside 5′-Triphosphate. UTP-EtNHDNP and CTP-EtNHDNP are both able to substitute for UTP and CTP, respectively, in the transcription of synthetic DNAs or T7 DNA (Figure 2A–C). Kinetic investigations of the transcription of T7 DNA in the presence of either UTP- or CTP-EtNHDNP were confined to the elongation process by initiating the polymerization with sufficiently high concentrations of CpA. This primer[3] initiates synthesis at promoter A1 (Minkley & Pribnow, 1973; Dausse et al., 1975; Smagowicz & Scheit, 1978). As shown in Figure 3, CpA effectively stimulates RNA synthesis when all other substrate concentrations are 5 μM. Employing [γ-^{32}P]ATP and [^{3}H]CTP, it was found that increasing concentrations of CpA resulted in increased incorporation of [^{3}H]CMP residues, which was paralleled by a decrease in the incorporation of [γ-^{32}P]ATP at the 5′ termini of RNA chains. This experiment indicates that CpA replaces ATP as a primer for RNA synthesis on promoter A1 of T7 DNA and does so at very low concentrations since the maximum stimulatory effect is obtained at only 10 μM CpA. K_m values for the pyrimidine derivatives were determined by making Lineweaver–Burk plots. It should be noted that the total rate of synthesis is much slower than under normal assay conditions since all substrate concentrations other than the derivatives are very low and the maximum velocity of the reaction is reduced for the derivatives. The DNPNHEt derivatives of UTP and CTP were found to

[3] Any nucleotide, oligonucleotide, or nucleotide analogue bearing a free 3′-OH group which binds to a binary complex DNA·RNA polymerase and can subsequently be phosphorylated by another incoming NTP is defined as a primer of RNA synthesis (Smagowicz & Scheit, 1977, 1978).

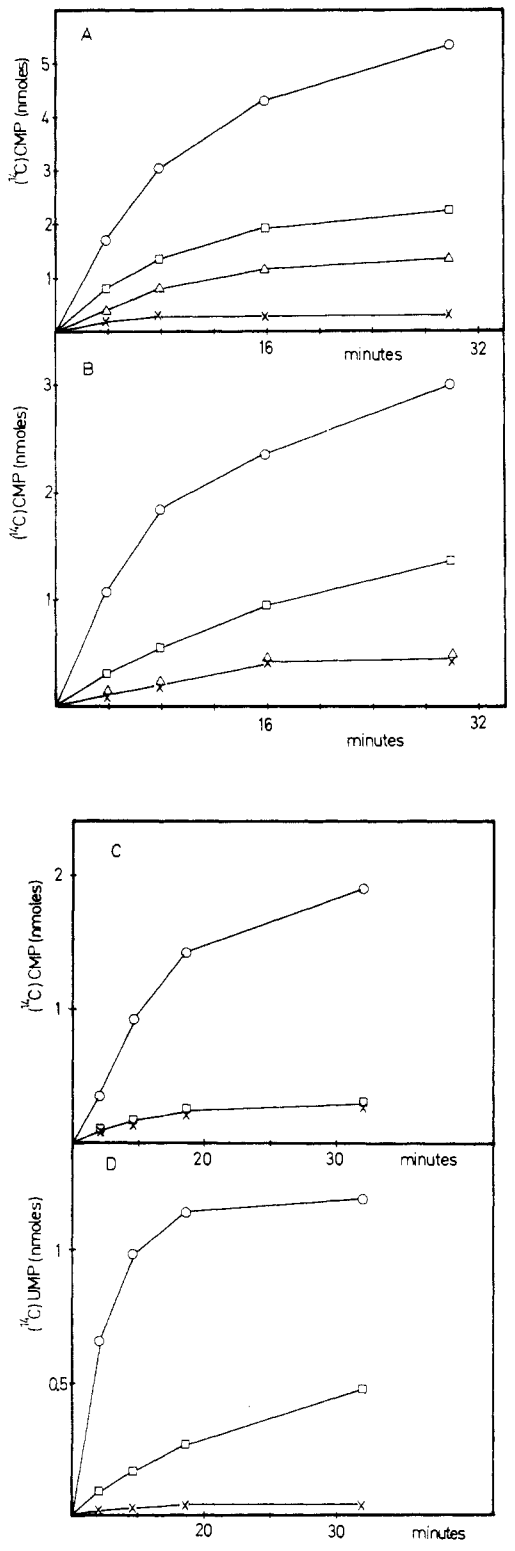


FIGURE 2: Transcription of T7 DNA in the presence of NTP esters. In a volume of 0.2 mL of buffer A, the incubation mixtures contained 0.4 pmol of T7 DNA, 4 pmol of RNA polymerase, and the following substrates (the numbers in parentheses following the nucleotides refer to the apparent velocity of the enzymatic reaction in units of nmol/8 min): (A) 0.15 mM ATP and GTP and 0.039 mM [^{14}C]CTP (10^5 cpm) with 0.039 mM of (○) UTP (2.95), (□) UTP-OCH_3 (1.15), (△) UTP-EtNHDNP (0.55), or (×) no UTP substrate; (B) 0.15 mM CpA and GTP, 0.04 mM UTP, and [^{14}C]CTP (10^5 cpm) with 0.04 mM (○) ATP (1.37), (□) ATP-OCH_3 (0.3), (△) ATP-EtNHDNP (no substrate), or (×) no ATP substrate; (C) 0.15 mM CpA and ATP, 0.05 mM UTP, and [^{14}C]CTP (10^5 cpm) with 0.05 mM (○) GTP (0.7), (□) GTP-EtNHDNP (0.05), or (×) no GTP substrate; (D) 0.15 mM CpG and ATP, 0.05 mM GTP, and [^{14}C]UTP (10^5 cpm) with 0.05 mM (○) CTP (0.87), (□) CTP-EtNHDNP (0.11), or (×) no CTP substrate.

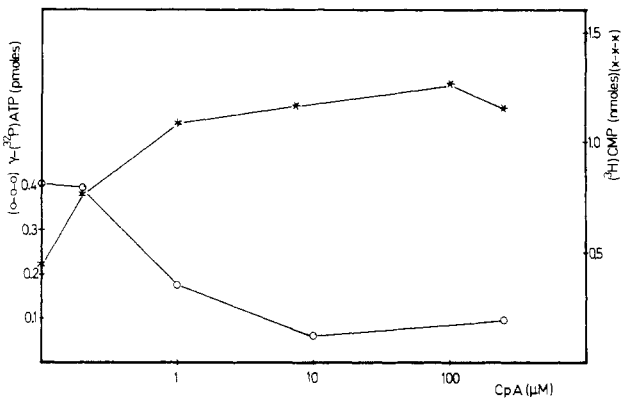


FIGURE 3: In 0.1 mL of buffer A, the incubation mixture contained 0.61 pmol of T7 DNA, 2.5 μg of enzyme, ATP, UTP, CTP, and GTP at 5 μM each, [^{3}H]CTP (50 cpm/pmol), [γ-^{32}P]ATP (5.9×10^3 cpm/pmol), and CpA in concentrations as indicated in the figure. DNA, Enzyme, and CpA were incubated 10 min at 37 °C. Synthesis was started by addition of substrates and allowed to proceed for 10 min at 37 °C. The reaction mixtures were subjected to acid precipitation as described under Materials and Methods.

be substrates with unfavorable kinetic parameters, K_m and V_{max} (table III). In comparison to K_m(UTP) = 16 μM, determined under identical experimental conditions (C. T. Kostia-Gromes and K.-H. Scheit, unpublished results), the K_m values for UTP- and CTP-EtNHDNP were found to be about 20 or 10 times higher, respectively (Table III). As can be seen from Figure 2 and its legend, UTP-OCH_3 also substitutes for UTP in the transcription of T7 DNA. Comparison under identical experimental conditions revealed a rate ratio, V(UTP)/V(UTP-OCH_3), of 2.4. ATP-OCH_3 substituted for ATP in the transcription of T7 DNA with a ratio, V(ATP)/V(ATP-OCH_3), of 4.6. Neither ATP- nor GTP-EtNHDNP (Figure 2A,C) functions as a substrate in the transcription of poly[d(A-T)] or T7 DNA. The utilization of UTP-EtNHDNP as a substrate in the transcription of poly[d(A-T)] led to the formation of DNPNHEt-PP and the respective polynucleotide. DNPNHEt-PP was identified from the reaction mixture by means of PEI–cellulose thin-layer chromatography, absorption spectroscopy, and enzymatic properties as well as comparison with an authentic sample of DNPNHEt-PP.

The controls for the base lines (absence of the respective nucleotides) and normal transcription (all normal nucleotides) are presented in Figure 2. Some of the base-line controls show a small increase of the counts with time, and the normal transcription controls are not all the same. This is because of the nature of the assay and the fact that the controls are done under separate, different conditions and are not identical experiments [different promoters and/or different starting conditions (see Figure 2)]. The assay will score even small oligonucleotides, and depending upon the promoters used, oligonucleotides of different lengths will be formed in the absence of correspondingly different nucleotides. Limited incorporation for the controls does not indicate cross-contamination of the nucleotides. Note that Figure 2A shows that ATP, GTP, and CTP are not contaminated more than 0.5% with UTP, and here we would expect the most sensitive test of contamination. UTP was purified by us.

Function of ADP, ATP, and *GTP Esters in Initiation of RNA Synthesis.* ATP-EtNHDNP failed to function as a substrate in the transcription of poly[d(A-T)]. However, the dinucleotide, DNPNHEt-pppApU, could be isolated from the reaction mixture by paper chromatography, and the isolation of this product on a preparative scale allowed its unequivocal identification from its absorption spectrum, the ratio of DNP

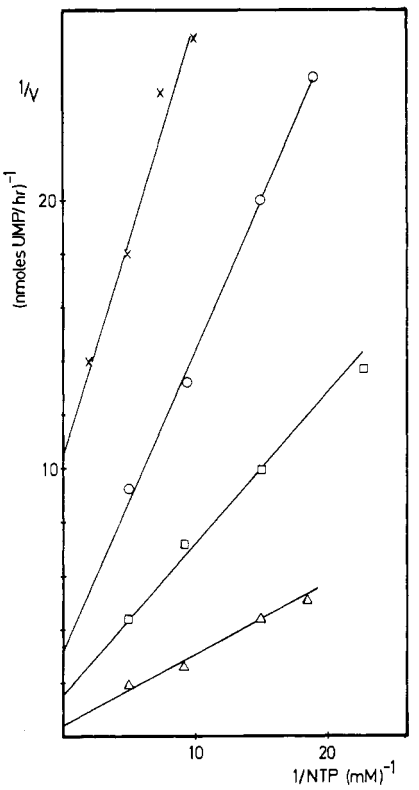


FIGURE 4: Steady-state kinetics of abortive initiation on T7 DNA. In 0.2 mL of buffer A, the incubation mixtures contained 0.4 pmol of T7 DNA, 4 pmol of RNA polymerase, 0.052 mM [^{14}C]UTP, and varying concentrations of ATP (△), ATP-EtNHDNP (□), ADP-EtNHDNP (○), or ATP-OCH_3 (×). Aliquots of the reaction mixture were taken at timed intervals and subjected to paper chromatography. The chromatograms were run twice in solvent 1 followed by solvent 2. The chromatograms were cut into 1-cm strips, which were measured for radioactivity in toluene-based scintillation fluid.

Table IV: Steady-State Kinetic Parameters of ATP Esters and ADP-EtNHDNP in Abortive Initiation on T7 DNA[a]

initiating nucleotide	K_m (mM)	V_{max} [nmol of UMP h^{-1} (pmol of enzyme)$^{-1}$]
ATP	1.1	4.83
ATP-EtNHDNP	0.4	0.353
ADP-EtNHDNP	0.4	0.69
ATP-OCH_3	0.16	0.097

[a] Data calculated from results depicted in Figure 4.

to [^{14}C]UMP, and its reaction with alkaline phosphatase and phosphodiesterase. Thus DNPNHEt-ATP is a primer for initiation, and the use of poly[d(A-T)] as a template results in a reaction similar to abortive initiation when this ATP derivative and UTP are substrates. The combination of ATP-OCH_3 and UTP leads to transcription of poly[d(A-T)] since ATP-OCH_3 is able to substitute for ATP in elongation.

The steady-state kinetic parameters of abortive initiation have been determined for promoters A1 and A3 of T7 DNA with ATP and UTP as substrates (Smagowicz & Scheit, 1977, 1978; McClure et al., 1978; McClure & Cech, 1978). We investigated the capability of ATP-EtNHDNP, ADP-EtNHDNP, and ATP-OCH_3 to substitute for ATP as a primer in abortive initiation, and the steady-state kinetic parameters of these derivatives during abortive initiation are depicted in Figure 4 and Table IV. All three compounds were found to have lower K_m's than ATP and significantly lower V_{max} values. ATP-OCH_3 represented the extreme case, with

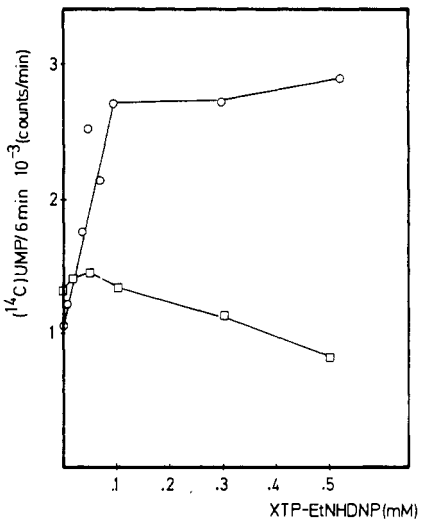


FIGURE 5: Stimulation of transcription of T7 DNA by ATP-EtNHDNP and GTP-EtNHDNP. In 50 μL of buffer A, the incubation mixture contained 0.14 pmol of T7 DNA, 2.4 μg of enzyme, ATP, GTP, and CTP at 5 μM each, 5μM [^{14}C]UTP (0.66×10^6 cpm/pmol), and ATP-EtNHDNP (○) as well as GTP-EtNHDNP (□) in concentrations as indicated. The mixture excepting substrates was incubated for 10 min at 37 °C. Synthesis, started by addition of substrates, was allowed for 6 min at 37 °C. Aliquots (20 μL) of the incubation mixture were subjected to paper-strip chromatography (Scheit & Faerber, 1975).

approximately 7-fold lower K_m and 50-fold reduced V_{max} compared to ATP. ATP-EtNHDNP was able to initiate RNA chains from promoters A1 and A3 in the transcription of T7 DNA. This was demonstrated in an experiment described in Figure 5 in which the rate of RNA synthesis was enhanced over 2-fold in the presence of ATP-EtNHDNP, with all other substrates at concentrations of 5 μM. This stimulation reached a plateau at 100 μM ATP-EtNHDNP. These results and those for abortive initiation lead to the conclusion that RNA chains intiated in the presence of saturating concentrations of ATP-EtNHDNP have the 5′ termini labeled with EtNHDNP.

RNA chains intiated from promoter A2 of T7 DNA start with the 5′ terminus pppGpC (Minkley & Pribnow, 1973; Dausse et al., 1975; Smagowicz & Scheit, 1978; T. A. Siebenlist, private communication). Stimulation from promoter A2 by GpC was found to be rather inefficient for unknown reasons. It is shown in Figure 5 that GTP-EtNHDNP stimulated transcription at low substrate concentrations to approximately 10% of the control without GTP-EtNHDNP. Saturation of this stimulatory effect on transcription was achieved at 50 μM GTP-EtNHDNP. Concentrations higher than 100 μM significantly inhibited transcription.

Discussion

All purine derivatives used in this study substitute for their respective unmodified nucleotides in the initiation of transcription. The base moiety retains the recognition properties of the natural purines, in spite of the modification of the nucleotides on the γ-phosphate position. Therefore the major requirements for an active primer function are retained with both the CH_3O and DNPNHEt esters of the purine derivatives.

The requirements of the elongation reaction appear to be more stringent regarding the modification of the terminal phosphate. Although all of the CH_3O derivatives are used as elongation substrates, compounds of the type NTP-EtNHDNP fall into two categories. The pyrimidine derivatives CTP- and UTP-EtNHDNP substitute for the corresponding natural

Table V: Properties in Transcription of ATP Analogues Derivatized on the Terminal Phosphate[a]

nucleotide	elongation	abortive initiation	initiation	inhibition of transcription
ATP	K_m = 15 μM[d]	K_m = 0.5 mM[b]	K_m = 150 μM[d]	K_i = 3.2 mM[e]
ATP-EtNHDNP	not used[k]	K_m = 0.4 mM[k]	K_m = 100 μM[k]	K_i = 1 mM[k]
ATP-OCH_3	fair[f,k]	K_m = 0.16 mM[k]	yes[k]	K_i = 1.7 mM[f]
ATP-γ-anilidate	good[j]	unknown	yes[j]	unknown
ATP-γ-AmNS	good[i]	unknown	yes[i]	unknown
ATP-γ-$(CH_2)_5$AmNS	not used[i]	unknown	unknown	unknown
adenosine 5′-tetraphosphate	very poor[f]	very good[c]	yes	K_i = 0.17 mM[f]
ATP-γ-F	poor[f]	unknown	unknown	K_i = 0.74 mM[f]
ATP-OPh	poor[f]	unknown	unknown	K_i = 1.8 mM[f]
ATP-γ-S	very good[g,h]	unknown	K_m = 150 μM	unknown
ADP	not used	K_m = 0.68[b]	unknown	unknown
AMP	not used	K_m = 2.5 mM[b]	unknown	unknown
adenosine	not used	K_m = 10 mM[b]		

[a] The superscript letters refer to the references which identify the literature source. Quantitative results have been given where they are available. Otherwise the qualitative description of the indicated authors has been used. [b] McClure et al. (1978). [c] McClure & Cech (1978). [d] Wu & Goldthwait (1969). [e] Rhodes & Chamberlin (1974). [f] Armstrong & Eckstein (1976). [g] Goody et al. (1972). [h] Reeve et al. (1977). [i] Yarbrough et al. (1979). [j] Grachev & Zaychikov (1974). [k] This work.

substrates, although with less favorable kinetic parameters. Their utilization as substrates involves the liberation of DNPNHEt-PP. However, the purine DNPNHEt derivatives did not function as substrates in elongation, although they are definitely incorporated at the 5′ position of the dinucleotides formed during an abortive initiation reaction. The experiments with ATP-EtNHDNP in transcription of poly [d(A-T)] and T7 DNA have also clearly shown that RNA chains can start with this purine derivative residue at the 5′ termini.

Apparently, the enzyme recognizes the pyrimidine NTP-EtNHDNP and purine NTP-EtNHDNP derivatives differently. This selection against the use of the EtNHDNP purine derivatives for elongation is probably due to steric constraints and/or additional interactions of the derivative with the transcribing complex through the DNP moiety. Since we have no decisive evidence that the conformation of the nuceotides about the γ-phosphate is different for the purine and pyrimidine derivatives, this could imply either that the enzyme has at least two binding sites for nucleotides, different for purines and pyrimidines, or that the substrate binding site can adopt more than a single conformation and that this conformation is dependent upon whether the site is occupied by a purine or a pyrimidine. In any case, the steric requirements for the triphosphate are not as stringent for initiation as they are for elongation.

The major transcriptional features of the EtNHDNP- and CH_3O-ATP derivatives are summarized in Table V together with other ATP γ-phosphate derivatives which have been previously reported. These analogues certainly support the conjecture that the capability of a nucleotide to participate in elongation is strongly dependent upon the structure of the 5′-phosphate moiety (Krakow et al., 1976). Quantitative information is not available for many of the compounds, but all the γ-phosphate ATP analogues are used as substrates for abortive initiation and/or as the initiating nucleotide in transcription. However, the γ-phosphate ATP derivatives differ most dramatically in their ability to support elongation. One might expect to find a correlation between the size of the chemical group attached to the γ-phosphate and the ability to substitute for ATP in transcription, but this does not seem to be the case. For instance, ATP-P^3-anilidate and ATP-P^3-AmNS both replace ATP in transcription very well, but ATP-OCH_3 and ATP-F are both poor substrates. On the other hand, ATP-OPh, in which the substituted group is of similar size as that in ATP-P^3-anilidate, is a poor substrate, and ATP-EtNHDNP is not used at all during elongation.

The total charge of the triphosphate also does not correlate well with the ability of an analogue to be used in elongation. ATP-P^3-anilidate, ATP-OCH_3, ATP-OPh, and ATP-EtNHDNP all have the same charge, and yet their ability to support elongation varies from good to none, respectively (Table V). It is also important to notice that for all the analogues with the exception of adenosine 5′-tetraphosphate, the inhibition of transcription in the presence of ATP is not very pronounced, and large concentrations of the nucleotides are needed to inhibit elongation. This is what is found for the normal nucleotides (Rhodes & Chamberlin, 1974) and shows that the inhibition of transcription by the analogues is not due to a strong competitive inhibition of ATP binding to the transcribing complex.

It is likely that more subtle effects are responsible for the substrate properties of these analogues such as the configuration of the nucleotide, especially the triphosphate moiety and its effect on the rest of the molecule. The conformation about the glycosidic bond strongly favors the anti configuration; however, the elongation nucleotide site of the transcribing complex tolerates fairly well modifications of the bases (Krakow et al., 1976). The importance of the complete triphosphate for elongation is especially apparent for AMP and ADP, and the configuration of the triphosphate of the nucleotide for activity has recently been emphasized by Armstrong et al. (1979). Substitution at the γ-phosphate could affect the ability of the phosphate chain to adopt a particular active configuration which is required for activity at the elongation nucleotide site. The complex of the divalent metal (Mg) with the triphosphate (Armstrong et al., 1979) could also be affected, especially at the β, γ position. Since the triphosphate structure of the purine is not required for the initiating nucleotide (or for abortive initiation), it is not surprising that substitutions at the γ-phosphate do not greatly affect the initiation reaction, since the part of the nucleotide which is involved in the chemical reaction is not adjacent to the triphosphate. The elongation nucleotide site may be more restrictive concerning the configuration of the phosphates.

If the triphosphate of the nucleotide must assume a specific configuration to be accepted as a substrate, why are the pyrimidine EtNHDNP derivatives elongation substrates whereas the purine EtNHDNP analogues are not used in elongation at all? One might expect the configuration of the triphosphate to be the same, or very similar, for both the purine and pyrimidine analogues. Interactions between the base and the substituent on the γ-phosphate would probably affect the configuration of the phosphate chain, and indeed, we have spectral evidence (small hypochromicity of the absorption

spectrum) which indicates some interaction of the dinitrophenyl chromophore of CTP-EtNHDNP and the base. Such spectral effects were not observed for the purine derivatives or for UTP-EtNHDNP. A similar situation has been found by Yarbrough et al. (1979) for the AmNS nucleotide derivatives. Although UTP-AmNS and ATP-AmNS are both good substrates for *E. coli* RNA polymerase, they report that only the pyrimidine derivative shows strongly quenched fluorescence. They suggest that this is due to the angle of the glycosidic bond which leads to more favorable stacking interactions between the fluorescent group and the pyrimidine base, as opposed to the purine base. However, under the assumption that such interactions apply to the EtNHDNP derivatives and that this could be responsible for the differences between the purine and pyrimidine derivatives, changes in the conformation of the nucleotides are assumed to take place fairly rapidly, within the turnover time of the enzyme. Thus it is not necessary that the substitution at the γ-phosphate stabilizes a particular configuration of the nucleotide in solution, but it could also destabilize a conformation of the nucleotide in the elongation site which is necessary for substrate activity.

Other evidence also indicates differences in the purines and pyrimidines during elongation. Armstrong & Eckstein (1976) reported that uridine tetraphosphate is a competitive inhibitor of both UTP and ATP, using an elongation assay of Rhodes & Chamberlin (1974). However, adenosine tetraphosphate is a competitive inhibitor only for ATP, not UTP for which it is an uncompetitive inhibitor. They interpreted this result in terms of the model proposed by Rhodes & Chamberlin (1974) such that adenosine tetraphosphate binds strongly only to one type of elongation site (C_T) but only weakly to the other elongation site (C_A) in a Ping-Pong mechanism. In terms of this model, the discrepancy between the EtNHDNP purine and pyrimidine derivatives in elongation could be due to topological differences in the elongation nucleotide site. Two substrate sites could either always be present or be induced by the template, which determines whether the incoming nucleotide is to be a purine or a pyrimidine substrate.

An indication that both the EtNHDNP and CH_3O ester derivatives interact with the transcriptional complex with higher affinity than the natural substrates comes from the results of the steady-state kinetic experiments. The kinetic parameters reveal a lower K_m for the adenine nucleotide esters than for ATP in initiation. This increased steady-state affinity is paralleled by a drastic reduction of V_{max}. Abortive initiation with poly[d(A-T)] as a template and ATP and UTP as substrates is a steady-state reaction, and the products pppApU and inorganic pyrophosphate are released relatively rapidly from the initiation complex. We have been able to establish that abortive initiation is not particularly sensitive to inhibition by the product pppApU, and therefore the affinity of pppApU appears to be low (W. J. Smagowicz and K.-H. Scheit, unpublished results). This suggests that the low V_{max} values for the purine nucleotide esters in abortive initiation could result from a high affinity of the product, DNPNHEt-pppApU, for the catalytic complex and/or a slower dissociation of the derivative product from the transcriptional complex. An alternative explanation would be a slower reaction due to unfavorable positioning of the 3′-OH of the ATP-EtNHDNP to be phosphorylated.

There is a discrepancy between the kinetic parameters of initiating triphosphate nucleotides determined from abortive initiation and the apparent affinity for the initiating nucleotides as determined by the stimulation of transcription at very low substrate concentrations. The K_m for ATP-EtNHDNP in abortive initiation was found to be 0.4 mM whereas the stimulation of transcription saturated at 100 μM ATP-EtNHDNP. This points to a significantly higher affinity (lower K_m) for ATP-EtNHDNP as an initiating nucleotide in transcription. Similarly, the K_m for CpA in primed abortive initiation on T7 DNA was determined to be 0.18 mM (Smagowicz & Scheit, 1978), but the apparent affinity for CpA as primer in transcription of T7 DNA at low substrate concentrations is 15 μM.

In summary, the EtNHDNP and CH_3O ester derivatives of the nucleotides reveal some subtle requirements for the structure of substrates for transcription by *E. coli* polymerase. In particular the 5′-end-labeling properties of ATP-EtNHDNP show great promise for use in the isolation of de novo synthesized RNA molecules by binding to DNP-specific antibodies.

Acknowledgments

Our thanks are due to Drs. T. M. Jovin, M. Temple, and G. Rhodes for helpful and stimulating discussions.

References

Armstrong, V. W., & Eckstein, F. (1976) *Eur. J. Biochem. 70*, 33.

Armstrong, V. W., Yee, D., & Eckstein, F. (1979) *Biochemistry 18*, 4120.

Chamberlin, M. J. (1974) *Enzymes, 3rd Ed. 10*, 333.

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

Chen, P. S., Toribara, P. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756.

Cramer, F., Gottschalk, E. M., Matzura, H., Scheit, K.-H., & Sternbach, H. (1971) *Eur. J. Biochem. 19*, 379.

Darlix, J. L., Fromageot, P., & Reich, E. (1971) *Biochemistry 10*, 1525.

Darlix, J. L., Fromageot, P., & Reich, E. (1973) *Biochemistry 12*, 914.

Dausse, J. P., Sentenac, A., & Fromageot, P. (1975) *Eur. J. Biochem. 57*, 569.

Goody, R. S., Eckstein, F., & Schirmer, R. H. (1972) *Biochim. Biophys. Acta 276*, 155.

Grachev, M. A., & Zaychikov, E. F. (1974) *FEBS Lett. 49*, 163.

Krakow, J. S., Rhodes, G., & Jovin, T. M. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M. J., Eds.) pp 17–67, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Livingstone, D. C., Dale, R. M. K., & Ward, D. C. (1976) *Biochim. Biophys. Acta 454*, 9.

Mangel, F. W., & Chamberlin, M. J. (1974) *J. Mol. Biol. 249*, 2995.

McClure, W. R., & Cech, C. L. (1978) *J. Biol. Chem. 253*, 8949.

McClure, W. R., Cech, C. L., & Johnston, D. E. (1978) *J. Biol. Chem. 253*, 8941.

Michelson, A. M. (1964) *Biochim. Biophys. Acta 91*, 1.

Minkley, E. G., & Pribnow, D. (1973) *J. Mol. Biol. 77*, 255.

Moffatt, J. G. & Khorana, H. G. (1961) *J. Am. Chem. Soc. 83*, 649.

Rackwitz, H. R., & Scheit, K.-H. (1977) *Eur. J. Biochem. 72*, 191.

Reeve, A. G., Smith, M. M., Pigiet, V., & Huang, R. C. C. (1977) *Biochemistry 16*, 4464.

Rhodes, G., & Chamberlin, M. J. (1974) *J. Biol. Chem. 249*, 6675.

Scheit, K.-H., & Faerber, P. (1975) *Eur. J. Biochem. 50*, 549.
Smagowicz, W. J., & Scheit, K.-H. (1977) *Nucleic Acids Res. 4*, 3863.
Smagowicz, W. J., & Scheit, K.-H. (1978) *Nucleic Acids Res. 5*, 1919.
Sternbach, H., Engelhardt, R., & Lezius, A. G. (1975) *Eur. J. Biochem. 60*, 51.
Thomas, C., & Abelson, J. (1966) in *Procedures in Nucleic Acids Research*, Vol. 1, pp 553, Harper and Row, New York.
van Broeckhoven, C., & de Wachter, R. (1978) *Nucleic Acids Res. 5*, 2133.
Ward, D. C., & Reich, E. (1972) *J. Biol. Chem. 247*, 705.
Ward, D. C., Reich, E., & Stryer, L. (1969) *J. Biol. Chem. 244*, 1228.
Wu, C. W., & Goldthwait, D. A. (1969) *Biochemistry 8*, 4458.
Yarbrough, L. R., Schlageck, J. G., & Baeughman, M. (1979) *J. Biol. Chem. 254*, 12069.
Zillig, W., Zechel, K., Halbwach, H. (1970) *Hoppe-Seyler's Z. Physiol. Chem. 351*, 221.

Affinity Labeling of Human Placental 17β-Estradiol Dehydrogenase and 20α-Hydroxysteroid Dehydrogenase with 5'-[*p*-(Fluorosulfonyl)benzoyl]adenosine†

Brian Tobias and Ronald C. Strickler*

ABSTRACT: Two pyridine nucleotide linked oxidoreductase activities, 17β-estradiol dehydrogenase and 20α-hydroxysteroid dehydrogenase, which were copurified from human placental cytosol as a homogeneous enzyme preparation, may represent dual activity by one enzyme. The affinity labeling nucleotide analogue 5'-[*p*-(fluorosulfonyl)benzoyl]adenosine, which binds at the cofactor site as a competitive inhibitor of NADH (k_i = 1.7 mM), simultaneously and identically inactivated both the 17β and 20α activities in a time-dependent and irreversible manner following pseudo-first-order kinetics. NADH and NAD^+ markedly protected both activities from inactivation, and the substrate steroids, estrone, estradiol, progesterone, and 20α-hydroxy-4-pregnen-3-one, conferred similar protection, though less than cofactor, against simultaneous loss of both activities. Stoichiometric studies indicated that 2 mol of affinity labeling nucleotide were bound per mol of completely inactivated enzyme dimer. The coincident and identical loss of both activities under all experimental conditions is further evidence that 17β-estradiol dehydrogenase and 20α-hydrosteroid dehydrogenase in human placental cytosol represent bifunctional, stereospecific, oxidoreductase activity at one active site on a single protein.

The usefulness of the affinity labeling nucleotide analogue 5'-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSA)[1] for the study of nucleotide-dependent enzymes has been clearly established (Pal et al., 1975; Wyatt & Colman, 1977; Colman et al., 1977). Furthermore, Sweet & Samant (1980) used this reagent to confirm the hypothesis that the 3α- and 20β-oxidoreductase activities of cortisone reductase (EC 1.1.1.53) represent bifunctional enzyme activity at a single active site. We have reported purification to homogeneity of a "protein fraction" from human placental cytosol which contains both 17β-estradiol dehydrogenase and 20α-hydroxysteroid dehydrogenase activities (Strickler & Tobias, 1980). Affinity alkylation of this "protein fraction" with 16α-bromoacetoxyprogesterone suggested that 17β-estradiol dehydrogenase and 20α-hydroxysteroid dehydrogenase represent dual enzyme activity at one active site (Strickler et al., 1981). We now describe further evidence that 17β-estradiol dehydrogenase and 20α-hydroxysteroid dehydrogenase are a function of a single enzyme active site.

Experimental Procedures

Materials. Purchased reagents and their suppliers were the following: nucleotides (NAD^+ and NADH), adenosine, 5'-[*p*-(fluorosulfonyl)adenosine hydrochloride, and iodoacetic acid from Sigma Chemical Co.; *p*-(fluorosulfonyl)benzoyl chloride from Aldrich Chemical Co.; Coomassie brilliant blue G-250 from Eastman Kodak Co.; estrone, 17β-estradiol, 17α-estradiol, progesterone, 20α-hydroxy-4-pregnen-3-one, and cholesterol from Sigma Chemical Co., which were found to be chromatographically pure and to have correct melting points; deuterated acetone from Stohler Isotope Chemicals; and inorganic chemicals and reagent grade organic solvents, which were not distilled prior to use, from Fisher Scientific Co. Glass-distilled deionized water was used for all aqueous solutions. Buffer A was 0.01 M potassium phosphate buffer, pH 7.0, containing 5 mM EDTA and 20% glycerol (v/v).

Methods. Melting points were determined in an Electrothermal apparatus from Fisher Scientific Co. Infrared spectra were obtained in potassium bromide pellets by using a Beckman Acculab IR-4. Nuclear magnetic resonance spectra were measured in deuterated acetone by using a Varian T-60. Ultraviolet absorption spectra were recorded on a Varian Cary 219 spectrophotometer. Protein concentrations were determined by the Coomassie blue method (Bradford, 1976).

Enzyme Preparation. A "protein fraction" containing both 17β-estradiol dehydrogenase and 20α-hydroxysteroid de-

† From the Department of Obstetrics and Gynecology, Washington University School of Medicine, St. Louis, Missouri 63110. *Received March 30, 1981.* Supported by the National Birth Defects Foundation Basil O'Connor Starter Grant 5-258.

[1] Abbreviations used: FSA, 5'-[*p*-(fluorosulfonyl)benzoyl]adenosine; NADH, β-nicotinamide adenine dinucleotide (reduced form); NAD^+, β-nicotinamide adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/81/0420-5546$01.25/0