Mechanism of Ribonucleic Acid Polymerase Action. Effect of Nearest Neighbors on Competition between Uridine Triphosphate and Uridine Triphosphate Analogs for Incorporation into Ribonucleic Acid*

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ABSTRACT: The consequence of permitting uridine triphosphate (UTP) and its analogs ribothymidine 5′-triphosphate (rTTP), 5-fluorouridine 5′-triphosphate (FUTP), and pseudouridine 5′-triphosphate (ψTP) to compete for incorporation in the presence of ribonucleic acid polymerase and templates of defined sequence, dAT, dAC:dTG, dAG:dTC, and poly (dA), has been studied. The values of K_m and V_{max} of the different precursors and the ratio of uridine monophosphate (UMP) to analog incorporated into RNA during competition experiments vary with the template.

Since each of the templates used permits only a single possible nearest neighbor of UMP or its analogs, observed preferences in the incorporation of UMP or the analog probably reflect differences in the base-stacking ability of the substrates with the nearest neighbors.

Complementary base pairing by hydrogen bonding is generally thought to be the predominant parameter governing the specificity of DNA-directed RNA synthesis (Schmidt, 1964). Nevertheless, there are indications of second-order effects which also play a role in this process. This was first shown in studies by Goldberg and Rabinowitz (1961) on the competition between UTP[1] and ψTP for incorporation at positions specified by AMP residues in the DNA template. They found that UTP and ψTP, present singly, were equally effective substrates for the RNA polymerase reaction and that each gave identical nearest neighbor frequencies; however, when both UTP and ψTP were present together and therefore allowed to compete in the incorporation, the nearest neighbor frequencies were altered in an interesting way. UMP was incorporated preferentially next to pyrimidines while ψMP was linked more frequently next to GMP residues.[2]

This is an unexpected result because if there was a bias for UTP or ψTP by the RNA polymerase, one would expect preferential incorporation of one precursor at all positions calling for UMP, *i.e.*, no change in the nearest neighbor frequencies. The implication of the Goldberg and Rabinowitz finding was that hydrogen-bonding specificity may not be the only parameter which permits discrimination between analogs. The experiments suggest that the nucleotide to which an incoming nucleotidyl residue becomes linked (*i.e.*, its nearest neighbor) influences the selectivity of incorporation. Kahan and Hurwitz (1962) tested other analogs of UTP, FUTP, BrUTP, and rTTP as substrates for RNA polymerase and found that with certain DNAs, particularly those with unusually high AT content, there was an alteration in the nearest neighbor frequencies. The latter experiments, however, did not examine the competition, *per se*, between analogs for their incorporation at different positions in the RNA sequence.

The availability of a series of high molecular weight polynucleotides of known sequence, dAT, dAC:dTG, dAG:dTC, and poly (dA), which provide a series in which each possible nearest neighbor of UMP or of an analog of UMP is defined by the strictly alternating sequence of bases in the template has led us to reex-

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[1] Abbreviations used: UTP, uridine 5′-triphosphate; ψTP, pseudouridine 5′-triphosphate; FUTP, 5-fluorouridine 5′-triphosphate; rTTP, ribothymidine 5′-triphosphate; CTP, cytidine 5′-triphosphate; GTP, guanosine 5′-triphosphate; ATP, adenosine 5′-triphosphate; CMP, cytidine monophosphate; similar abbreviations have been used for the corresponding nucleoside 5′-monophosphates; dAT, deoxyadenylate:deoxythymidylate copolymer; poly (dA), polydeoxyadenylate; dAC:dTG, the helical complex of alternating deoxycytidylate:deoxyguanylate copolymer; dAG:dTC, the helical complex of alternating deoxyadenylate:deoxyguanylate copolymer and alternating deoxythymidylate:deoxycytidylate copolymer; rAU, riboadenylate:ribouridylate copolymer; rCU, ribocytidylate:ribouridylate copolymer; rGU, riboguanylate:ribouridylate copolymer; rArT, riboadenylate:ribothymidylate copolymer; rAFU, riboadenylate:ribofluorouridylate copolymer; rAψ, riboadenylate:ribopseudouridylate copolymer.

[2] Goldberg and Rabinowitz normalized all nearest neighbor frequencies to the frequency of ApU and Apψ and, therefore, the preferences for UMP or ψMP next to AMP residues cannot be deduced from their paper.

amine the phenomena described by Goldberg and Rabinowitz. It will be shown that the K_m and V_{max} of the precursors and the relative incorporation of UMP and its analogs into RNA during competition experiments vary with the nearest neighbor.

Materials and Methods

Materials. RNA polymerase prepared from *Escherichia coli* was fraction IV with a specific activity of 2000–3000 when assayed as previously described (Chamberlin and Berg, 1962). The 2′,3′-*O*-isopropylidine derivative of 5-fluorouridine was a gift of Dr. Charles Heidelberger. Thymine riboside was obtained from Cyclo Chemical Corp.; pseudouridine, A grade (approximately 66% pseudouridine C), was purchased from Calbiochem; [^{32}P]H_3PO_4 was a product of New England Nuclear Corp.; [^{3}H]UTP and [α-^{32}P]UTP were obtained from Schwarz BioResearch; [α-^{32}P]UTP was also synthesized as described below.

Synthesis of α-^{32}P-Labeled Nucleoside Triphosphates. The 2′,3′-*O*-isopropylidine derivatives of thymine riboside, pseudouridine, and uridine were prepared by the method of Hampton (1961) except that in some cases triethylammonium bicarbonate in 50% methanol was used to elute the blocked derivatives from Dowex 1 (HCO_3^-) rather than NH_4HCO_3 in 50% methanol. (The triethylammonium bicarbonate has the advantage of being more easily removed when the products are dried *in vacuo*.) The 2′,3′-*O*-isopropylidine nucleosides were treated with [β-^{32}P]cyanoethyl phosphate (Pfitzner and Moffatt, 1964) (sp act. 200–1000 mc/mmole) using conditions described by Wehrli *et al.* (1965) for the synthesis of [α-^{32}P]adenosine 5′-monophosphate. The isolation of free nucleoside monophosphates was modified only in that the removal of the β-cyanoethyl group was accomplished by heating at 65° for 30 min in concentrated ammonia. The α and β anomers of ψMP, ψMP B, and ψMP C were resolved by chromatography on Dowex (HCO_3^-) as described by Chambers *et al.* (1963) except that 0.14 M triethylammonium bicarbonate, rather than ammonium bicarbonate, was used as eluent. The 5′-mononucleotides were converted to the corresponding triphosphates by the method of Hoard and Ott (1965) and purified on DEAE HCO_3^- as described by Wehrli *et al.* (1965). The isolated nucleoside triphosphates were converted to their potassium salts by passage through Dowex 50 (K^+). Unlabeled nucleoside triphosphates were prepared as described above.

Polydeoxynucleotides. Poly dAC:dTG and poly dAG:dTC were prepared and isolated essentially as described by Wells *et al.* (1965) by replicating authentic primers (obtained from Dr. H. G. Khorana) with DNA polymerase from *E. coli.* Replication of dAG:dTC and to a lesser extent dAC:dTG was quite sluggish and was accompanied by the synthesis of variable amounts of poly (dAT) when a highly purified fraction of the polymerase (fraction VIII of Richardson *et al.* (1964)) was used. The addition of 4–6 units of a cruder fraction (fraction IV) to 350 units of fraction VIII per

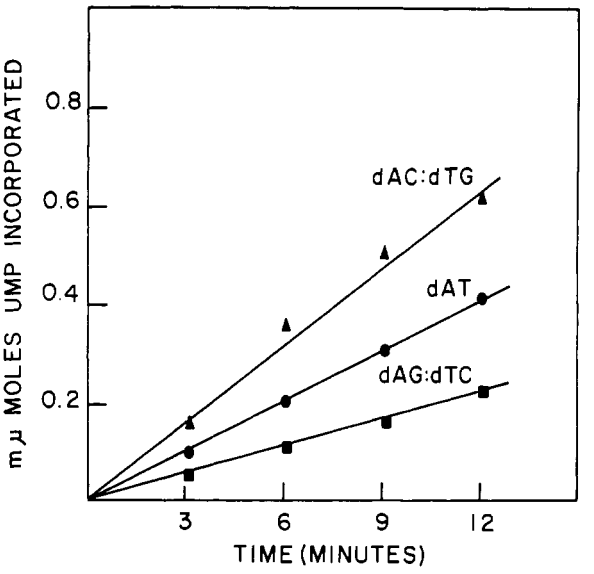

FIGURE 1: The incorporation of UMP into rAU, rCU, and rGU as a function of time at 15°. The reaction conditions are described under Methods. The initial concentration of [α-^{32}P]UTP (sp act. 5 × 10^3 cpm/mμmole) was 100 μM.

ml of reaction mixture resulted in a rapid replication of dAG:dTC and dAC:dTG with little or no formation of dAT (as measured by the ability of the products to prime for poly (rAU) synthesis). Under these conditions a tenfold replication of input primers was usually obtained. In order to avoid any ambiguities that might arise owing to the presence of even small amounts of undesired polynucleotides in the dAG:dTC and dAC:dTG preparations, all experiments in which the incorporation of UTP or its analogs was followed were performed in the presence of only the two relevant triphosphates. Thus, with dAG:dTC as template only CTP and UTP and/or its analogs were added to the RNA polymerase reaction mixtures and with dAC:dTG as template only GTP and UTP and/or its analogs were added.

Poly (dAT) copolymer was prepared according to Schachman *et al.* (1960). Poly (dA) was the gift of Dr. T. Jovin.

Incubation Conditions and Assay. The standard system contained the following components at the concentrations indicated in a final volume of 100 μl: Tris-HCl buffer (pH 8.0), 40 mM; $MgCl_2$, 1 mM; 2-mercaptoethanol, 12 mM; dAT, dAC:dTG, dAG:dTC, or poly (dA), 75–100 μM in nucleotide; ATP, CTP, or GTP, 250–350 μM; RNA polymerase, 14 units. UTP and/or its analogs were varied as shown in each experiment (see Results). RNA polymerase was added last. When incubation times of less than 10 min were used, the tubes containing the reaction mixture were preincubated for 2 min at 15° (see Results) before addition of the enzyme. Reactions were stopped by addition of 2 ml of cold 2 N HCl containing sodium

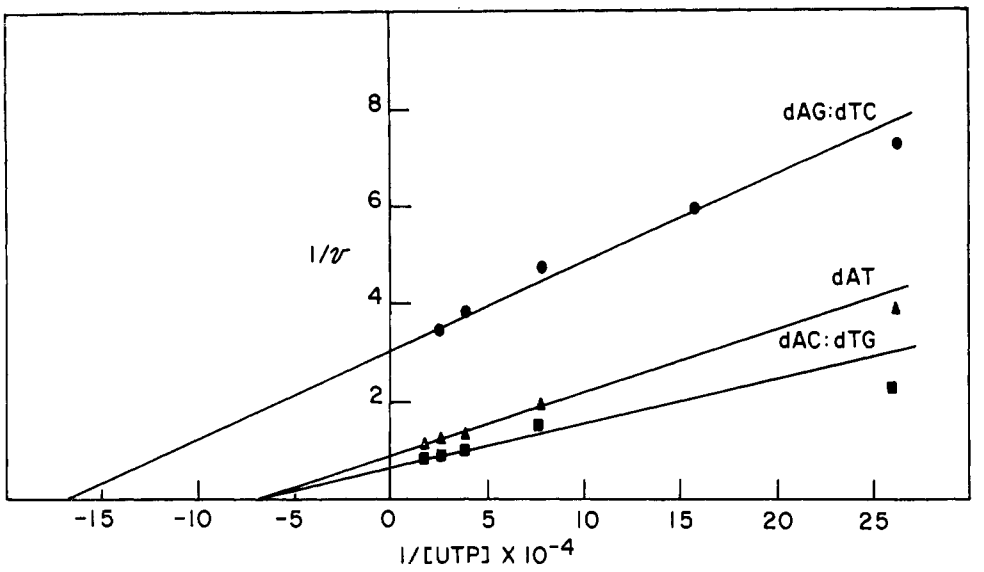

FIGURE 2: Lineweaver–Burk plots of the rate of UMP incorporation with dAG:dTC, dAC:dTG, and dAT as templates. Incubation time 5 min; other conditions as described under Methods.

pyrophosphate at a concentration of approximately 0.1 M. The cold acid-insoluble materials were collected on 2.4-cm Whatman GF/C glass-fiber filters and washed with ten 3-ml aliquots of cold 2 N HCl containing 0.1 M sodium pyrophosphate followed by two 3-ml aliquots of ethanol. The dried disks were counted in a Nuclear-Chicago Mark I liquid scintillation counter under 5 ml of a solution containing 2,5-diphenyloxazole (4.0 g) and dimethyl-*p*-bis[2′-(5′-phenyloxazolyl)]benzene (0.10 g) in toluene (1 l.).

Results

The incorporation of [^{32}P]UMP into rAU, rCU, and rGU as a function of time at 15° is shown in Figure 1. The temperature of 15° was chosen for these studies in order to extent the time range over which the reaction rate was linear with time. At 27°, using the same concentrations of reactants, the rate was linear for only 2–5 min. As can be seen in Figure 1, the rate of UMP incorporation varies with the template. Thus, dAC:dTG supports the greatest rate followed by dAT and dAG:dTC approximately in the ratio of 3:2:1.

Determination of the Apparent K_m and Relative Maximal Velocity for UTP and Its Analogs as a Function of Template. The values of the apparent K_m and V_{max} for UTP, FUTP, rTTP, and ψTP were determined with dAT, dAC:dTG, and dAG:dTC as templates. Figure 2 shows typical results obtained for UTP with these templates when the data were plotted according to Lineweaver and Burk (1934). With poly (dA) as template, only the data with UTP and ψTP gave straight-line plots; with rTTP or FUTP as substrates, Lineweaver–Burk double-reciprocal plots did not yield straight lines although a straight line could be obtained by plotting $1/v$ *vs.* $1/S^2$.

The values for K_m and the relative maximal velocities of UTP and its analogs with each of the templates are summarized in Table I. It can be seen that the value of K_m for each of the substrates varies with the template used. In general, for each of the substrates tested the values of K_m obtained with dAT or dAC:dTG as template are quite similar, while the values obtained with dAG:dTC as template are significantly lower. When poly (dA) was used as template the values of K_m for UTP and ψTP were significantly higher than with the other templates.

The relative maximal velocities vary both as a function of template and as a function of substrate when the same template is used. For example, with dAT as template the maximal velocity of rArT synthesis is 32% greater than rAU synthesis, while the rate of rAFU and rAψ synthesis is approximately half that of rAU. Similarly, rTMP incorporation is faster than UMP incorporation into rUG (dAC:dTG as template) but the two are incorporated at equal rates into rUC (dAG:dTC as template). ψMP incorporation was slower than that of UMP when dAT or dAG:dTC were templates, but it was faster when dAC:dTG was template. With poly (dA) as template the maximal velocity of poly (ψ) synthesis was only one-fifth that of poly (U) synthesis.

Competition Experiments. An alternative approach to determining the effect of nearest neighbors on the incorporation of UMP and its analogs was to examine the competition between [^{3}H]UTP and α-^{32}P-labeled analog when each was present in the reaction mixture either at its K_m concentration or eight times its K_m concentration (at or near saturation). The reactions

TABLE I: The Apparent K_m and Relative V_{max} of UTP and Its Analogs with Various Templates.[a]

	App K_m and Rel V_{max} When the Following Are Used as Template:							
	dAT		dAC:dTG		dAG:dTC		Poly dA	
Substrate	K_m	Rel V_{max}	K_m	Rel V_{max}	K_m	Rel V_{max}	K_m	Rel V_{max}
UTP	18	1.00	15	1.00	6	1.00	135	1.00
rTTP	112	1.32	110	1.32	16	0.96		
FUTP	61	0.49	67	0.72	9	0.29		
ψTP	9	0.43	10	1.36	6	0.72	45	0.19

[a] Reaction conditions are those described in Methods and Figure 2. Values for K_m (micromolar) and relative V_{max} are the average of at least three independent determinations.

were allowed to proceed for 1–3 min and the amount of each nucleotide incorporated into RNA was determined. Under these conditions less than 1% of the initial substrate was consumed. Since each precursor was present at its K_m or at a saturating concentration proportional to its K_m, the ratio of the incorporation of UMP to analog monophosphate should provide a direct measure of the relative ease with which these substrates serve as nucleotidyl donor to each of the possible nucleotides at the growing point. The results of these experiments are given in Table II. When UTP and rTTP compete, rTMP is preferentially incorporated regardless of the neighboring nucleotide. When UTP and FUTP compete, UMP is preferentially incorporated next to AMP and CMP residues, while little or no preference exists between the two substrates for incorporation next to GMP residues. In the case of UTP-ψTP competition, UMP was preferentially incorporated next to AMP but ψMP is incorporated more rapidly next to GMP residues; UMP was preferentially incorporated next to itself or ψMP. Little preference was observed between UMP or ψMP if incorporation occurred next to CMP residues.

TABLE II: Ratio of [^{3}H]UMP:α-^{32}P Analog Monophosphate Incorporated When Both Substrates Were Present at K_m or at Saturating Concentrations as a Function of Template.[a]

Substrate	Template	K_m (μM)	Ratio of Incorp of [^{3}H]UMP: α-^{32}P Analog[b] When Both Substrates Were Present at K_m	Ratio of Incorp of [^{3}H]UMP: α-^{32}P Analog[b] When Both Substrates Were Present at $8 \times K_m$
rTTP	dAT	112	0.66	0.79
	dAC:dTG	110	0.61	0.61
	dAG:dTC	16	0.64	0.46
FUTP	dAT	61	1.40	1.10
	dAC:dTG	67	0.96	0.75
	dAG:dTC	9	2.20	1.50
ψTP	dAT	9	1.35	1.33
	dAC:dTG	10	0.49	0.39
	dAG:dTC	6	1.00	0.80
	Poly (dA)	45	2.80	2.70

[a] Reaction conditions are those described in Methods. The times of incubation were 1 min when dAT, dAC:dTG, or poly (dA) served as template and 3 min when dAG:dTC served as template. The specific activities of the substrates were: [^{3}H]UTP, 63 cpm/$\mu\mu$mole; [α-^{32}P]rTTP, 34 cpm/$\mu\mu$mole; [α-^{32}P]-FUTP, 127 cpm/$\mu\mu$mole; and [α-^{32}P]ψTP, 58 cpm/$\mu\mu$mole. The minimum incorporations observed after correction for background and a no-enzyme blank were: UMP, 3.5 $\mu\mu$moles; rTMP, 10 $\mu\mu$moles; FUMP, 1.0 $\mu\mu$mole; and ψMP, 4.0 $\mu\mu$moles. [b] Each value represents the average of at least two determinations.

Since the pK_a's of ψTP and of UTP differ (the pK_a of the former is pH 8.97 and that of the latter is pH 9.25 (Ofengand and Shaefer, 1965)), it is possible that in this case the observed preferences are due to differences in charge on the two bases. One might expect, therefore, that the ratio of UMP:ψMP incorporation would vary with pH. The ratio of UMP:ψMP incorporated at pH values (Tris-HCl buffer, 15°) of 7.33, 7.66, 8.01, 8.69, 9.48, and 9.81 was determined and was found to be virtually invarient with each of the templates tested, *i.e.*, dAT, dAC:dTG, and dAG:dTC. Similarly, the addition of increasing amounts of KCl to the point of complete inhibition of the reaction caused no change in the observed ratio of UMP:ψMP incorporated for each of the templates tested. These results suggest that differences in pK_a with resultant differences in charge on these substrates are probably not the basis for the preferences found.

Discussion

In the experiments reported here we have examined the effect of different nearest neighbors on the specificity with which an adenosine residue in the polydeoxynucleotide template directs the incorporation (by RNA polymerase) of either UMP or one of its analogs.

TABLE III: Preferences in the Incorporation of UMP and Its Analogs into RNA When Adding to Different Neighbors.

	Nearest Neighbor	Preferences
1	A, G, C, U	T > U
2	A	U > FU or ψ
3	G	ψ > U ≶ FU
4	C	ψ = U ≫ FU
5	U or ψ	U ≫ ψ

To do this the double-stranded DNA-like polymers dAT (Schachman *et al.*, 1960), dAC:dTG, and dAG:dTC (Wells *et al.*, 1965) and the single-stranded polymer dA were used as templates to direct the synthesis of the complement of the A-containing strand, *i.e.*, the alternating polymers rUA, rUG, rUC, and the homopolymer rU and the corresponding polymers with analogs of U. Each polymer provides a different nearest neighbor for the entering UMP or UMP analog and we measure the preference between UMP and the analog during the polymerization process.

Our results confirm and extend the earlier observations of Goldberg and Rabinowitz (1961). Although UTP, ψTP, FUTP, and rTTP form base pairs almost exclusively with the A of the template, their relative rates of incorporation differ depending upon the nucleotide to which they become attached. Table III summarizes the preferences we have observed. U is incorporated more efficiently next to A than is FU or ψ, but U is incorporated less efficiently than ψ next to G. On the other hand, UTP and ψTP are equally good substrates for incorporation next to C and both are far better than FU. When U is the nearest neighbor, U is preferentially incorporated compared to ψ.

These results cannot be explained solely by differences in the hydrogen-bonding potential of the different substrates. If this were the case one would expect that there would be the same preference for one substrate over another regardless of the base adjacent to the A of the template. For example, it might be that the preference in the incorporation of T relative to U regardless of the neighbor is due to the formation of stronger hydrogen bonds between T and A than between U and A (Szer, 1965). On the other hand, T also shows stronger base-stacking interactions than does U (Shugar and Szer, 1962; Szer, 1965) and as suggested below this may account for the preference of T over U. Because the competition between substrates was examined at their respective K_m concentrations, one can eliminate differences in the affinity of the enzyme for the different analogs as accounting for the observed preferences.

A more reasonable explanation for the preferences between U, ψ, and FU with different neighbors is that base-stacking interactions between the incoming base and the adjacent base can influence the incorporation of one of the two competing species. It may be that given two precursors with nearly equal hydrogen-bonding specificity and stability, the one in which the base-stacking interaction with the adjacent base is strongest will be preferentially incorporated. From studies of the hypochromicity (Michelson, 1963), circular dichroism (Brahms *et al.*, 1967), and optical rotatory dispersion (Warshaw and Tinoco, 1965, 1966) of dinucleotides and the self-association studies of Ts'o *et al.* (1963), it has been concluded that U stacks poorly with any of the other three bases. Unfortunately, no data are yet available comparing the base-stacking interactions of U and its analogs. If the preferential incorporations we have observed result from differences in base stacking, one would expect to find, for example, differences in these measurable parameters between ApU and Apψ or GpU and Gpψ or CpU and CpFU.

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