Heteropolynucleotide Synthesis with Terminal Deoxyribonucleotidyltransferase*

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ABSTRACT: The terminal deoxyribonucleotidyltransferase from calf thymus that is known to catalyze the polymerization of a single deoxyribonucleoside 5'-triphosphate has been found to copolymerize all combinations of two, three, and four such triphosphates. Whereas 2'-deoxyadenosine 5'-triphosphate (dATP) is most effectively utilized alone, 2'-deoxyguanosine 5'-triphosphate (dGTP) is best in all mixtures. Melting curves have been run for the derived heteropolymers, leading to the conclusion that they are almost entirely single stranded.

Preliminary investigation of the dA,T polymer has shown that a great variety of lengths of thymidylate sequences are present.

The synthesis of polydeoxyribonucleotides using terminal deoxyribonucleotidyltransferase (addase) from calf thymus, a deoxyribonucleoside 5'-triphosphate, and an initiator which is an oligodeoxyribo-5'-nucleotide has been described by Bollum *et al.* (1964) and Hayes *et al.* (1966). The present experimental work was designed to determine whether more than one deoxyribonucleoside 5'-triphosphate could be simultaneously incorporated into polymer and, if so, what the characteristics of the reactions and products might be.

Materials and Methods

Addase was obtained from calf thymus glands and was purified by the procedure of Yoneda and Bollum (1965); its specific activity was 10 μmoles of dATP[1] incorporated into polymer per milligram of protein per hour with $d(pT)_6$ as initiator. Deoxyribonucleoside 5'-triphosphates were obtained ^{14}C labeled from Schwarz Bio-Research, Inc., and unlabeled from Calbiochem, Nutritional Biochemicals Corp., and P-L Biochemicals, Inc. Whatman P-11 phosphocellulose was obtained from H. Reeve Angel Co. and was processed according to the procedure described for DEAE-cellulose by Ratliff *et al.* (1964). The initiator $d(pT)_6$ was synthesized according to Khorana and Vizsolyi (1961).

The reaction mixture (1.5 ml), containing 40 mM potassium phosphate (pH 7.0), 8 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 60 μg of addase, 1.0 μmole of total nucleoside 5'-triphosphate, and 0.01 μmole of $d(pT)_6$, was incubated at 37°. Each single-, the mixture of all four-, and all combinations of two- and three-nucleoside 5'-triphosphates were used. Several complete sets of the required 32 reactions were run with one of the nucleoside 5'-triphosphates being labeled in each of the reactions. For each radioactivity assay an aliquot of 0.1 ml was removed, and the acid-insoluble product was counted on glass fiber disks according to Hayes *et al.* (1966) except that, in the case of dT, heat-denatured calf thymus DNA was used as carrier in the precipitation step. Duplicate reactions were deproteinized by passage through phosphocellulose (100 mg/mg of protein, equilibrated with 0.05 M potassium phosphate, pH 6.9) and dialyzed against four 1-l. changes of SSC (0.15 M NaCl–0.015 M sodium citrate, pH 7.0) (Marmur and Doty, 1962). Also processed in this manner were dAT:dAT, the double-stranded alternating copolymer of dAMP and dTMP (Schachman *et al.*, 1960), and two calf thymus DNA-polymerase double-stranded products, dA:dT from dA with dTTP (Bollum, 1964) and dA,T:dA,T from dA,T with dTTP and dATP.

Thermal hyperchromicity measurements (absorbance *vs.* temperature profiles) were determined in SSC. Absorbance was measured with a Beckman DK-1 spectrophotometer equipped with a repeating slide wire. Sample temperature was measured with a glass-enclosed thermocouple dipping into the sample through a silicone stopper in the sample cuvet. Signals were recorded simultaneously on a Moseley *X–Y* recorder. The *Y*-axis (absorbance) range was adjusted so that the ratio of absorbance to initial absorbance ($A:A_0$) was plotted directly. Sample temperature was changed at 0.4°/min by circulating Prestone through a jacketed cuvet holder from a Tamson

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[1] Abbreviations used: SSC, standard saline citrate buffer; dATP, dCTP, dGTP, and dTTP are 5'-triphosphates of 2'-deoxyadenosine, 2'-deoxycytidine, 2'-deoxyguanosine, and thymidine, respectively; dpT and $d(pT)_6$ are monomer and hexamer of 5'-thymidylic acid; dpTp is thymidine 3',5'-diphosphate; $d(pA)_{\overline{130}}$ is a mixed polydeoxy-5'-adenylic acid with average length of 130 units; otherwise, polymer notation is that of Inman and Baldwin (1962) except that the $d(pT)_6$ segment common to the polymers is omitted from the abbreviated designations.

TABLE I: Incorporation and Hyperchromicity Data for Addase-Derived Homo- and Heteropolymers.

Polymer	Incorporation of Nucleotides (%)				Av Rate of Hyperchromicity (%/deg)
	dAMP	dCMP	dGMP	dTMP	
dA	51 (98)[a]	–	–	–	0.36
dC	–	0.7 (46)	–	–	42[b]
dG	–	–	15 (19)	–	0.04
dT	–	–	–	2.3 (34)	−0.03
dA,C	7.8 (70)	6.8 (89)	–	–	0.26
dA,G	56 (100)	–	65 (100)	–	0.35
dA,T	22 (42)	–	–	15 (51)	42[b]
dC,G	–	11 (74)	26 (88)	–	86[b]
dC,T	–	1.6 (51)	–	2 (28)	0.03
dG,T	–	–	21 (50)	6.2 (19)	0.08
dA,C,G	15 (54)	15 (81)	32 (98)	–	0.18
dA,C,T	5.4 (57)	4.8 (73)	–	5.7 (72)	0.07
dA,G,T	26 (83)	–	40 (97)	15 (71)	0.27
dC,G,T	–	7.5 (52)	20 (81)	5.4 (32)	0.10
dA,C,G,T	16 (57)	14 (78)	31 (94)	10 (64)	0.10

[a] 1 hr (24 hr). [b] T_m, °C.

TABLE II: Radioactivity Measurements in Experiment with Poly dA,T Labeled Triphosphate.

Isolated 3′-Deoxynucleotide	Reaction 1, dATP-α-^{32}P			Reaction 2, dTTP-α-^{32}P		
	Sequence	Cpm	Fraction	Sequence	Cpm	Fraction
Tp	TpA	45,482	0.613	TpT	38,532	0.657
Ap	ApA	28,742	0.387	ApT	20,108	0.343
Sum		74,224	1.000		58,640	1.000

heating bath equipped with a motor-driven temperature regulator.

Polymer depurination and hydrolysis to give 3′,5′-terminal diphosphates of pyrimidine sequences were accomplished according to Burton and Petersen (1960). Chromatographic analysis for size distribution was performed by passing the product mixture through a Bio-Gel P-60 column (1 × 120 cm, 0.047 M triethylammonium bicarbonate) that had been calibrated with $d(pA)_{\overline{130}}$, $d(pA)_8$, dpTp, and dpT.

Experimental and Results Section

After preliminary experimentation had established that more than one deoxyribonucleoside 5′-triphosphate could be utilized simultaneously in the reaction with $d(pT)_6$ to make acid-insoluble product, all possible combinations were tried. The per cent mononucleotide incorporation for both 1- and 24-hr reaction times is shown in Table I including, for comparison, our data for the cases involving only one nucleoside triphosphate at a time (Bollum *et al.*, 1964).

To investigate the extent and intensity of base interaction in the 24-hr polymers, melting curves (Marmur and Doty, 1962) were determined. The data for the average slope of the curve over the temperature range investigated are shown in Table I. The temperatures corresponding to the maxima of the calculated differential curves (change in hyperchromicity per degree *vs.* temperature) were designated as the characteristic melting temperature (T_m) for the cases showing a clear inflection in their melting curves. This procedure has the advantage over the half-height method (Marmur and Doty, 1962) of being independent of slope, if present, of the initial and final portions of the melting curve. Differential curves for dA,T, dC,G, dA,T:dA,T, dAT:dAT, and dA:dT are shown in Figure 1.

The polymer d(A-8-^{14}C),T was degraded to adenine-8-^{14}C and a mixture of 3′,5′-terminal diphosphates of thymidylate oligomers (Burton and Petersen, 1960). After treatment to remove adenine, only 0.3% of

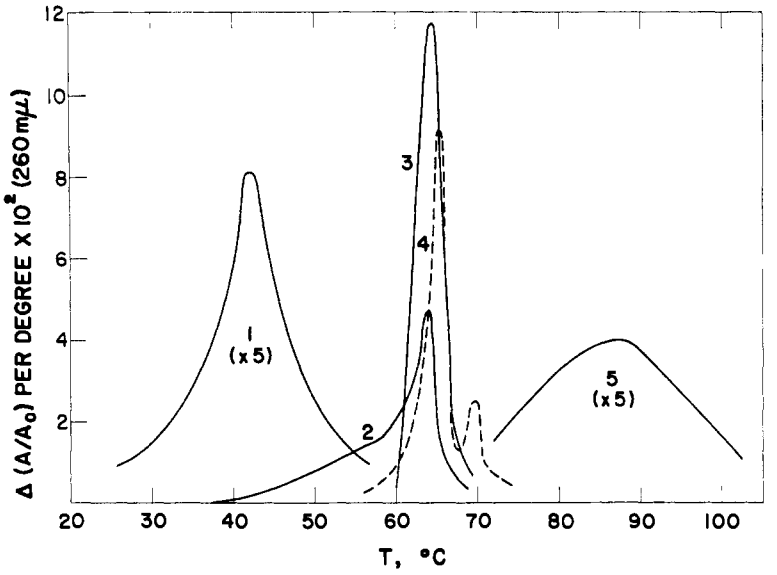

FIGURE 1: Differential thermal hyperchromicity curves for dA,T (1), dA,T:dA,T (2), dAT:dAT (3), dA:dT (4), and dC,G (5). Curves 1 and 5 are plotted with ordinate values five times the experimental values.

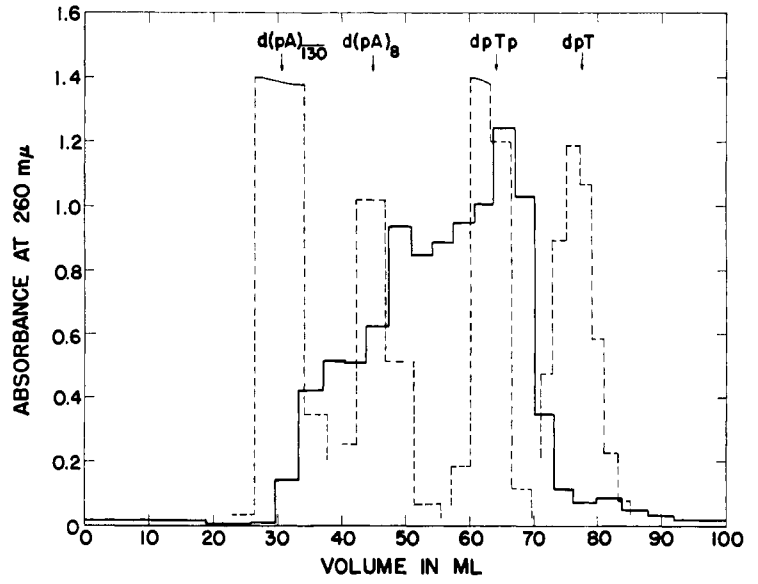

FIGURE 2: Gel filtration (Bio-Gel P-60) chromatographic elution pattern of the Burton and Petersen (1960) degradation product of dA,T (heavy lines). In the background (dashed lines) are calibration elution patterns for $d(pA)_{\overline{130}}$, $d(pA)_8$, dpTp, and dpT.

the original ^{14}C activity remained with the thymidylate oligomers. Gel filtration chromatography then gave the size-distribution profile of Figure 2, which is superimposed upon a composite calibration profile. Nearest neighbor studies were done on the polymers d(A-5′-^{32}P),T and dA,(T-5′-^{32}P) according to the procedure of Josse *et al.* (1961), and the results are given in Table II.

Discussion

Bollum *et al.* (1964) established that the addase reaction proceeds with dATP at a far greater rate (50 times) than with dCTP, dGTP, or dTTP. Our study clearly confirms that dATP is the superior substrate when used singly. Whereas dCTP and dTTP are both only slightly utilized in 1 hr, they reach 30–50% in

24 hr. The result is different with dGTP, which reaches 15% in 1 hr and then shows only a slight increase to 19% in 24 hr. The low rate of polymerization of dGTP is thought to be due to aggregation of continuous stretches of deoxyguanylate either intra- or intermolecularly, preventing further addition of the dGTP to the polymer (Ratliff and Hayes, 1967). In all combinations of dGTP, the rate and extent of addition of dGTP are higher than with dGTP alone. This is to be expected if the low yield of polymer with dGTP alone is due to deoxyguanylate aggregation. If that is the case, then addition of another deoxynucleoside triphosphate to prevent long runs of deoxyguanylate should prevent aggregation and increase the efficiency of dGTP as a substrate.

The major observation in this study is that all the combinations of triphosphates will function simultaneously as substrates. In these cases the 24-hr incorporation is always considerably higher than the 1-hr result, and dGTP is the superior substrate. For the combination reactions, the total extent of incorporation appears to depend more strongly on purine content than on Watson and Crick (1953) hydrogen-bonded pairs. The efficacy of purines may lie in the hydrophobic purine stacking observed by Howard *et al.* (1966) and by Huang and Ts'o (1966).

In the thermal hyperchromicity data, there is evidence of weak Watson and Crick (1953) interaction in dA,T and dC,G (Figure 1). The observations with dC are similar to those of Inman (1964), who investigated polydeoxycytidylate separated from the Radding *et al.* (1962) dC:dG homopolymer pair. Otherwise, it is generally true that higher purine content gives greater total hyperchromicity.

The differential curves for thermal hyperchromicity shown in Figure 1 include the only two addase heteropolymers that gave inflections in their integral curves: dA,T and dC,G. Also plotted in Figure 1 is the presumably doubly stranded complementary duplex synthesized using dA,T as template in the calf thymus DNA-polymerase reaction (Bollum, 1964). Comparison of this curve with those of two standard deoxyadenylate–thymidylate polymers, dAT:dAT (Schachman *et al.*, 1960; Marmur and Doty, 1962) and dA:dT (Bollum, 1964, 1965), shows that there is A:T interaction in dA,T:dA,T. The slow rise in the differential melting curve of dA,T:dA,T over the temperature range below its T_m may be an indication of some partially double-stranded loops present in the original dA,T. Calf thymus DNA-polymerase has an absolute requirement for a single-stranded template (Yoneda and Bollum, 1965). The fact that this polymerase used dA,T as a template shows that much of the dA,T is single stranded. Information thus far obtained on nucleotide sequence shows that thymidylate and deoxyadenylate sequences in dA,T exist in a variety of lengths in the same strand, establishing, at least in this case, the random heteropolymeric nature of the new polymers.

Efforts presently in progress are completion of both nearest neighbor frequency analyses (Josse *et al.*, 1961) on all the polymers and the Burton and Petersen (1960) degradations on the purine–pyrimidine combination polymers, followed by anion-exchange chromatography analysis of pyrimidine chain frequencies. It will also be interesting to see how nucleotide sequence in the polymers may be altered by varying the proportions of different nucleoside triphosphates.

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