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Limited Enzymatic Addition of Deoxyribonucleotide Units onto Chemically Synthesized Oligodeoxyribo-5′-nucleotides*

F. N. Hayes, V. E. Mitchell, R. L. Ratliff, and D. L. Williams

ABSTRACT: The oligomer-initiated polymerization of deoxyribonucleotide units catalyzed by terminal deoxyribonucleotidyl transferase has been limited by reaction stoichiometry to the formation of short polymers. The product distributions conform reasonably well to Poisson statistical theory in these cases. However, in the self-limiting polymerization of deoxyguanylate units, the distribution was very sharp.

Oligo- and polydeoxyribonucleotides of varied and known structure are miniature models of DNA suitable for studies of its chemical and biological properties. Such compounds have been synthesized by exclusively chemical procedures (Jacob and Khorana, 1965) and also by combinations of chemical and enzymatic methods (Bollum *et al.*, 1964; Byrd *et al.*, 1965; Hayes *et al.*, 1966).

The calf thymus derived enzyme, terminal deoxyribonucleotidyl transferase (hereafter referred to as addase), has been used to effect syntheses of polydeoxyribonucleotides with 100 or more monomer units of one kind (Bollum *et al.*, 1964) or with mixed sequences of different kinds of monomers (Ratliff *et al.*, 1967). This reaction involves repetitive grafting of mononucleotide units from a deoxyribonucleoside 5′-triphosphate onto an oligodeoxyribonucleotide initiator beginning at its terminal 3′-hydroxyl. Before addase had been separated from calf thymus DNA polymerase (Bollum *et al.*, 1964), Bollum (1962) had used the partially purified mixture of these enzymes to add just a few nucleotide units onto various initiators.

We have used addase freed of DNA polymerase (Yoneda and Bollum, 1965) to effect synthesis of short polynucleotides by using small ratios of deoxyribonucleoside 5′-triphosphate to initiator. In the case using dGTP,[1] an additional self-limiting effect on length has already been noted (Ratliff and Hayes, 1967) at greater than elevenfold ratios of triphosphate to initiator. Distributions of products formed during a reaction have been compared with Poisson theory.

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[1] Abbreviations used: oligodeoxynucleotides are designated by the usual capital letters followed by subscripts representing the number of units. Examples are T_6 hexamer of 5′-thymidylic

Materials and Methods

The procedure of Khorana and Vizsolyi (1961) was used to synthesize T_4-^3H, T_5-^3H, T_6, T_6-^3H, T_{10}, and $T_{\bar{16}}$-^3H. The last was an incompletely resolved mixture, primarily T_{16} but containing also some T_{15} and T_{17}. The addase-derived products $A_{\bar{97}}$ and $(T_6$-$^3H)G_{\bar{11}}$ were synthesized according to Hayes *et al.* (1966) and Ratliff and Hayes (1967). P-L Biochemicals was the source of dATP, dCTP, dGTP, and dTTP, while dCTP-2-^{14}C was procured from Schwarz BioResearch, Inc. Triphosphates were quantitatively analyzed for purity by paper chromatography (all were greater than 90%). Amounts used in reactions were corrected for actual triphosphate content. Whatman P-11 phosphocellulose was obtained from H. Reeve Angel Co., and DEAE-cellulose and Bio-Gels P-2, P-60, and P-100 came from Bio-Rad Laboratories.

Addase was isolated from calf thymus glands and purified by the procedure of Yoneda and Bollum (1965); its specific activity was 13 μmoles of dATP incorporated into polymer/mg of protein per hr (13,000 units/mg) with T_6 as initiator. Addase recovery from completed reactions was accomplished by flowing the reaction mixture through a column of phosphocellulose (100 mg/mg of protein) which retains the addase and passes the nucleotide components. Subsequent removal of addase was with 0.3 M potassium phosphate (pH 6.9). Addase had been previously shown by Hayes *et al.* (1966) to be free of endonucleolytic activity. For this study, in control-corrected reactions with 5′-thymidylic acid and with T_3, it was demonstrated that addase had no detectable phosphatase activity under the conditions used here.

Gel filtration chromatography to separate polymers from unused initiator and triphosphate was carried out on a Bio-Gel P-60 column (9.1 cm^2 × 40 cm, 0.047 M triethylammonium bicarbonate, 1 ml/min, ascending). Desalting was accomplished with a Bio-Gel P-2 column (13.9 cm^2 × 49 cm, water). Anion-exchange chromatography was done on a DEAE-cellulose column (0.8 cm^2 × 15 cm, 0.5 ml/min) at pH 7.5 with 2 l. of a linear 0.005–0.5 M triethylammonium bicarbonate gradient or at pH 2.5 with the same volume of a linear 0.005–0.5 M lithium chloride in 3 mM hydrochloric acid gradient.

Polymer length was determined by the isotopic dilution of labeled initiator (Hayes *et al.*, 1966). Counting was done with both Packard Model 3324 and Beckman Model CMP-100 scintillation spectrometers setting the ^{14}C channels to have zero counting efficiency for tritium (Hayes, 1966). All tritium counts were corrected for radioactive decay. Counting results were converted to absolute disintegration rates using internal standards of water-^3H and urea-^{14}C. Polymer quantities in mono-micromoles were assigned as equal to analyses for esterified phosphorus (Petersen *et al.*, 1962), in micro-gram-atoms, assuming that each nucleotide unit had associated with it one phosphate group.

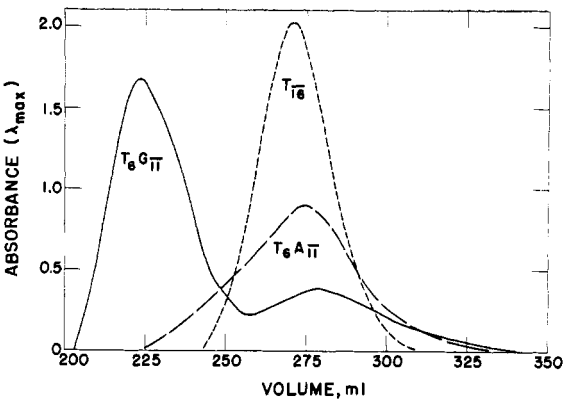

FIGURE 1: Elution profiles from Bio-Gel P-100 of $T_6G_{\bar{11}}$, $T_6A_{\bar{11}}$, and $T_{\bar{16}}$.

Synthesis of $(T_6$-$^3H)A_{\bar{11}}$. A 19-ml reaction in potassium phosphate (0.12 M, pH 6.9), containing 1.0 μmole of T_6-^3H (1.0×10^7 dpm/μmole), 8 μmoles of dATP, 3.5 mg of addase, 200 μmoles of magnesium chloride, and 21 μmoles of 2-mercaptoethanol, was incubated at 37° for 15 hr. After recovery of addase the product was fractionated on Bio-Gel P-60. The polymer peak contained 7.0 mono-μmoles and analyzed for $T_6A_{\bar{10.7}}$.

Product Length Distribution Analyses of $T_6A_{\bar{11}}$ *and* $T_6G_{\bar{11}}$. An LKB Recychrom column (8.0 cm^2 × 100 cm) was packed with Bio-Gel P-100 (96 cm, 0.047 M triethylammonium bicarbonate, 14 ml/hr, ascending, 30 min/tube) and calibrated, giving the following volumes to the peak absorbance of the elution profile: $A_{\bar{97}}$, 222 ml; T_{16}, 271 ml; T_{10}, 310 ml; and dGTP, 476 ml. Subsequently, under essentially identical conditions, 6.9 mono-μmoles of $(T_6$-$^3H)G_{\bar{11}}$ and then 4.4 mono-μmoles of $(T_6$-$^3H)A_{\bar{11}}$ were fractionated on this column. The profiles of these last two runs plus that of $T_{\bar{16}}$ are shown in Figure 1. The tubes from each of the two mixed-oligomer runs were segregated such as to give seven fractions continuously across the whole profile. Each fraction was analyzed for radioactivity and phosphorus content from which were calculated the average chain lengths. The number of units added for the first and seventh fractions of the entire $T_6G_{\bar{11}}$ double peak were 11.3 and 10.1, with the other five results in between. However, with the single $T_6A_{\bar{11}}$ peak, the similarly arrived at values ranged from 18.7 down to 4.4. To adequately present the great difference between these two results, the frequency distribution curves of product from each run were calculated from the fraction data and are shown in Figure 2; the points indicate the theoretical Poisson distribution numbers for the case with

acid, T_4C_2 hexamer containing four thymidylate units at the 5′ end and two deoxycytidylate units at the 3′ end, and $(T_6$-$^3H)G_{\bar{11}}$ heptadecamer with six tritium-labeled thymidylate units at the 5′ end and a distribution of deoxyguanylate units with mean of eleven at the 3′ end. 5′-Triphosphates of deoxyadenosine, deoxycytidine, deoxyguanosine, and thymidine are dATP, dCTP, dGTP, and dTTP. 5′-Terminally dephosphorylated oligomers are represented with a hydroxyl at the left end of the above convention: HO-T_4 thymidylate tetramer with a free 5′-hydroxyl group. The monomicromole is a micromole of monomer units contained in a polymer.

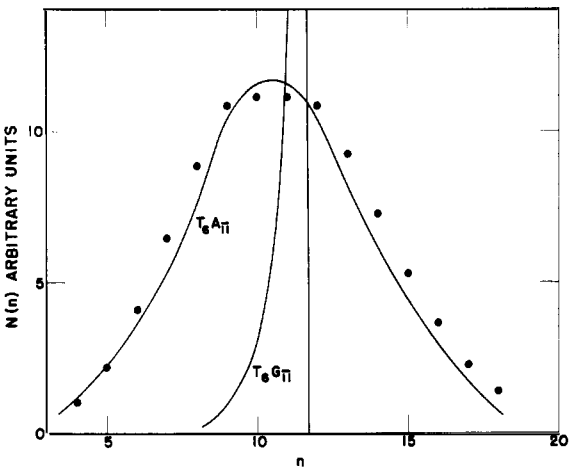

FIGURE 2: Experimental and theoretical frequency distributions of polymer lengths where n is the number of units added onto the T_6 initiator and $N(n)$ is the relative number of moles. Solid curves are $T_6A_{\overline{11}}$ and $T_6G_{\overline{11}}$; points are the Poisson theory numbers.

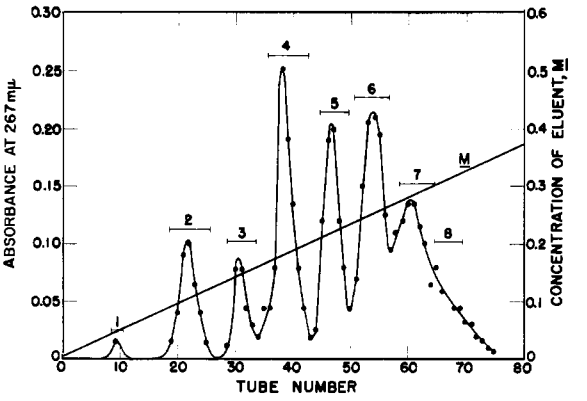

FIGURE 3: Ion-exchange chromatography of products from the reaction of T_4 with dCTP-2-^{14}C followed by dTTP. The numbers above the profile are the fraction numbers.

a mean of eleven additions. The number-average total lengths of the entire $T_6G_{\overline{11}}$ and $T_6A_{\overline{11}}$ recovered from the Bio-Gel P-100 column were 17.3 and 16.9, respectively.

Reaction Sequence to Add One C Unit Followed by One T Unit onto T_4-^3H. A 40-ml reaction mixture, containing 2.08 μmoles of T_4-^{3}H (1.64×10^6 dpm/mono-μmole), 2.20 μmoles of dCTP-2-^{14}C (2.67×10^4 dpm/μmole), 16.2 mg of addase, 0.4 mmole of magnesium chloride, 56 μmoles of 2-mercaptoethanol, and 12 mmoles of potassium chloride, was adjusted to pH 7.0 with dilute potassium hydroxide. There was no orthophosphate in this mixture, since the addase had been dialyzed previously against 0.3 M potassium chloride. The reaction was incubated at 15° for 6 hr, which was judged to be more than enough time for all the dCTP to react; whereupon dTTP (2.14 μmoles) was added, and the incubation was allowed to continue for another 17 hr.[2] The reaction was then diluted with 40 ml of water and passed through phosphocellulose, from which 1.8×10^5 units of addase (85% of original activity) were later recovered. The deproteinized eluate was desalted on Bio-Gel P-2. The sample was then fractionated on DEAE-cellulose at pH 7.5. Individual tubes were spectrophotometrically assayed at 267 mμ (Figure 3), and fractions 4–8 were individually pooled. After concentration and removal of the volatile salt, each fraction was analyzed for carbon-14, tritium, phosphorus, and ultraviolet absorbance. A separate chromatographic run with T_4-^{3}H on the same DEAE-cellulose column under identical conditions to the above gave a profile with minor peak, identified as HO-T_4,[3] followed by T_4 (94%). Both peaks were superimposable on the profiles of fractions 2 and 4, respectively. Therefore, material in fraction 4 had five negative charges and, because of the even spacing, probably all fractions contained products with one more charge than the fraction number. Fractions 1–3 contained nucleoside mono- and diphosphates present as minor contaminants in the original triphosphates, plus any unused portions of the triphosphates and the HO-T_4. Data for these fractions are not reported here, since they were an unimportant part of the product.

Fractions 4–8 were considered to contain products, according to the pattern of Figure 4, that underwent zero, one, or two additions at each of the two reaction steps, members of the series $T_4C_xT_y$. According to the radioactive-labeling pattern, the four T units at the 5′ end were tritium labeled, any C units were carbon-14 labeled, and any T units in addition to the four from the initiator were unlabeled. Specific radioactivity values were calculated for all nine compounds and compared with the observed, giving the found mole per cent initiator T and C data in Table I. The monomicromole data for fractions 4–8 came from phosphorus analyses.

There were compounds in fraction 4 other than T_4 that both lowered the mole per cent initiator T from 100 to 92 and increased the mole per cent C from 0 to 2.9. A likely source was from addition to the contaminating HO-T_4 of two nucleotide units, at least one being C, giving compounds with a charge of five at pH 7.5 and a mole per cent initiator T of 67. From this it was possible to calculate a composition for the

[2] This part of the reaction was run considerably longer than the first part because of the observation (unpublished data) that dTTP has a smaller incorporation rate constant than dCTP.

[3] Since this observation, we have encountered cases of a small extent of terminal dephosphorylation in other 5′-thymidylate oligomers that have been stored frozen. The direct cause of this has not yet been discovered. Ralph and Khorana (1961) have noted similar phenomena.

TABLE I: Composition and Yield Data for the Series $T_4C_xT_y$.

Fraction	Mono-μmoles	Mole % Initiator-T Calcd	Mole % Initiator-T Found	Mole % C Found		Mole % C Calcd	μmoles	Mole % Poisson Prediction	Mole % Found
4	2.60	100	92	2.9	T_4	0	0.50	16	27
5	2.24	80	79	13	T_5	0	0.16	16	9
					T_4C	20	0.29	16	16
6	3.06	67	69	21	T_6	0	0.0	8	0
					T_4CT	17	0.35	16	19
					T_4C_2	33	0.16	8	9
7	2.19	57	56	23	T_4CT_2	14	0.12	8	6
					T_4C_2T	29	0.19	8	10
8	0.59	50	51	25	$T_4C_2T_2$	25	0.07	4	4

mixture of fraction 4: T_4, 0.50 μmole; HO-T_4CT, 0.08 μmole; and HO-T_6, 0.03 μmole.

Fraction 5 had its composition calculated, from being a binary mixture, and was rechromatographed on DEAE-cellulose at pH 2.5. Two compounds were isolated with mole per cent values. *Anal.* Calcd for T_4C: 64. Found: 65. Calcd for T_5: 36. Found: 35. The mole per cent C in the isolated T_4C determined from specific radioactivity was 21, calcd 20.

Fraction 6 was rechromatographed on DEAE-cellulose at pH 2.5, giving only two peaks identified by charge and carbon-14 specific activity (in mole per cent C) as T_4CT. *Anal.* Calcd for T_4CT: 17. Found: 17. Calcd for T_4C_2: 29. Found: 33. Since fraction 6 proved to be a binary mixture, its composition was amenable to calculation from the mixture data (in mole per cent). *Anal.* Calcd for T_4CT: 75. Found: 69. Calcd for T_4C_2: 25. Found: 31.

The composition of fraction 7 was calculated from the carbon-14 specific activity as mole per cent values; calcd for T_4CT_2: 38; calcd for T_4C_2T: 62. From the mixture composition data the number of micromoles of each product was calculated and entered in Table I. The final two columns in Table I are the values for mole per cent of total from the yield data and from Poisson statistics with two successive single-addition steps, each limited to no more than two additions.

Discussion

It has been shown here in two examples that the reaction between an oligodeoxyribo-5′-nucleotide initiator and a deoxyribonucleoside 5′-triphosphate catalyzed by addase can be made to produce short polymers by using small ratios of triphosphate to initiator. The reactions were processed in different ways: the product from T_6-^{3}H and dATP and, for comparison, the polymer T_6G_{11} were crudely fractionated into seven contiguous parts of the polymer profile resulting from gel filtration; the product from T_4-^{3}H that was reacted firs with dCTP-2-^{14}C and then with dTTP was chromatographically separated into products of different charge.

The addase reaction, if free running and continuously competitive, should be a statistical process leading to formation of a distribution of product lengths; moreover, the distribution should be described by Poisson statistics. If m is the mean number of added nucleotide units, that is equal to the actually reacting mole ratio of triphosphate to initiator, and x is any one of the individual numbers added, the mole fraction of polymer with x units added should fit the Poisson expression: $e^{-m}m^x/x!$ However, self-limiting effects during an addase reaction such as product aggregation could give a much sharper distribution of product lengths. The comparison of T_6A_{11} with T_6G_{11} is most revealing. As shown in Figure 1, the apparent molecular size distribution exhibited by gel filtration chromatography was broader for T_6G_{11} than for T_6A_{11} and uniquely bimodal for the former. Chain-length analysis across the distribution that resulted from gel filtration gave the frequency distribution profiles of

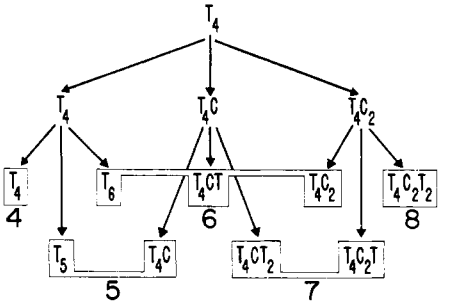

FIGURE 4: Reaction scheme for the conversion of T_4 to the series $T_4C_xT_y$. The large numbers below the boxed groupings of products are the fraction numbers from Figure 3.

Figure 2, showing that $T_6A_{\overline{11}}$ was in close agreement with the broad Poisson prediction for 11 additions by ranging clearly from 5 to 17 additions, where $T_6G_{\overline{11}}$ was sharply confined to the region of ten to twelve additions. Evidently, aggregation not only limited the polymerization of deoxyguanylate units onto T_6 but also produced considerable clustering of $T_6G_{\overline{11}}$ molecules, as shown in the gel filtration profile. This is in agreement with the observations and conclusions of Ralph *et al.* (1962) with deoxyguanylate oligomers.

The reaction of T_4-^{3}H with 1 molar equiv of dCTP-2-^{14}C followed by another equivalent of dTTP gave a product mixture that was either completely fractionated into individual polymers or partially fractionated and then calculated for individual composition by quantitative radioisotopic anlaysis leading to the results in Table I.

According to the reaction scheme in Figure 4, the intermediates at the end of the first step were two new oligomers, T_4C and T_4C_2, along with unreacted T_4. By separately summing the Poisson predictions and the found mole per cent values in the last two columns of Table I for the sets of three products resulting from each of these intermediates, it can be seen that the Poisson and experimental data are in good agreement. *Anal.* Calcd for T_4, T_4C, and T_4C_2: 40, 40, and 20, respectively. Found: 36, 41, and 23, respectively. The agreement at the end of the reaction for the six compounds resulting from intermediates T_4C and T_4C_2 is also good, but the three final products arising *via* the sequence of no reaction with dCTP-2-^{14}C followed by zero, one, or two additions of dTTP, giving the compounds T_4, T_5, and T_6, had mole per cent yields quite different from the predicted, being predominantly T_4. One mechanism by which this may have happened is a rearrangement of the addase enzyme surface during the long period of no reaction in the first step such that the altered T_4–addase complex then went through the motions of reacting with dTTP (that is, effectively competing with the T_4C and T_4C_2 complexes) without efficient condensation.

The limited addition of deoxyribonucleotide units onto a chemically synthesized oligodeoxyribo-5′-nucleotide with addase will allow synthesis of short polymers that can be used as initiators in further addase reactions to make block copolymers. Thus, considerably more information content can be built into synthetic polydeoxyribonucleotides than from one-step, single-monomer, addase reactions.

The addase reaction to add a single unit onto a short initiator is not competitive in yield with stepwise chemical synthesis because of the statistical distribution of products formed. Bollum (1962) suggested the use of monomers with blocked 3′-hydroxyl groups to limit the reaction to a single addition. Thus far we have been unable to effect participation of $O^{3'}$-acetyldeoxyadenosine 5′-triphosphate in the addase reaction. The synthesis and study of other similar potential substrates are in progress.

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