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Incorporation Efficiency of Small Oligo-5′-nucleotide Initiators in the Terminal Deoxyribonucleotide Transferase Reaction*

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ABSTRACT: Radioactive oligodeoxyribo-5′-nucleotides ($d(pA)_2$, $d(pT)_2$, $d(pA)_3$, $d(pT)_3$, $d(pC)_3$, and $d(pC)_4$) have been used as initiators in the terminal deoxyribonucleotide transferase reaction under a variety of conditions with 2′-deoxyadenosine 5′-triphosphate (dATP) to give single-stranded polydeoxyribonucleotides with labeled 5′-end sequences. Per cent incorporation of initiator and 2′-deoxyadenosine 5′-monophosphate (dAMP) and also polymer length have been determined for each product. Conditions which increased initiator incorporation were low temperature, high phosphate concentration, and high enzyme specific activity. The initiators showed the preference order for incorporation: tetramer > trimer > dimer and A > T > C, and also influenced the rate of dAMP incorporation in the order A > T > C.

A reaction catalyzed by terminal deoxyribonucleotidyl transferase (hereafter referred to as addase) involves repetitive grafting of mononucleotide units from a deoxyribonucleoside 5′-triphosphate onto the 3′-terminal hydroxyl function of a growing single-stranded polymer (Bollum, 1960, 1962). The reaction requires an oligodeoxyribo-5′-nucleotide as an initiator, and the first addition takes place on its 3′-hydroxyl. The minimum size for a competent initiator is trimer (Bollum *et al.*, 1964).

We have isolated and purified addase (Yoneda and Bollum, 1965) in order to synthesize single-stranded polydeoxyribonucleotides from a variety of trimer initiators (Williams *et al.*, 1965) to serve as templates in biological information transfer studies. We have investigated the incorporation of radioactive initiators into polymers in order to understand the factors controlling the efficiency of utilization.

Materials and Methods

Oligo-5′-thymidylic-2-^{14}C acids, $d(pT\text{-}2\text{-}^{14}C)_n$,[1] and

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[1] Abbreviations used in this work: dATP, 2′-deoxyadenosine 5′-triphosphate; dAMP, 2′-deoxyadenosine 5′-monophosphate; $d(pA)_2$ and $d(pA)_3$, dimer and trimer of dAMP; $d(pT)_2$ and $d(pT)_3$, dimer and trimer of 5′-thymidylic acid; $d(pC)_3$ and $d(pC)_4$, trimer and tetramer of 2′-deoxy-5′-cytidylic acid; mono-μmole, a micromole of monomer units contained in a polymer; *E*, per cent incorporation of initiator; *Y*, per cent incorporation of dAMP; $\bar{n}$, average number of monomer units in polymer; $\bar{x}$, average number of added dpA units; PPO, 2,5-diphenyloxazole; POPOP, 2,2′-*p*-phenylenebis(5-phenyloxazole).

oligo-5′-thymidylic-^{3}H acids, d(pT-^{3}H)$_n$, were prepared by the procedure of Khorana and Vizsolyi (1961). Oligodeoxy-5′-cytidylic-^{3}H acids, d(pC-^{3}H)$_n$, were prepared by the procedure of Khorana *et al.* (1961). Oligodeoxy-5′-adenylic-^{3}H acids, d(pA-^{3}H)$_n$, were synthesized by deoxyribonuclease (DNAase) I degradation of polydeoxyadenylic-^{3}H acid (Bollum *et al.*, 1964) prepared by addase reaction of d(pA)$_3$ with a 300-fold molar ratio of dATP-^{3}H. The thymidylic and deoxycytidylic oligomers were purified by column chromatography on DEAE-cellulose, first at pH 7.5 followed by pH 5.0.

Addase was obtained from calf thymus glands[2] according to the procedure of Yoneda and Bollum (1965). Common to the standard assay and reaction mixtures were 10 μM initiator, 1 mM dATP, 8 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.05 M pH 6.9 potassium phosphate buffer, and incubation at 35°. Unless otherwise stated, all reactions were made up in this way, which should ideally add 100 dAMP residues onto each molecule of initiator (1000 μM dATP/10 μM initiator). The addase, with d(pT)$_6$ as initiator, had a dATP utilization rate of 7×10^3 and 1.5×10^4 units/mg (1 unit = 1 nmole of dAMP into polymer/hr) in two different preparations.

Addase assays used dATP-8-^{14}C (Schwarz BioResearch, Inc.) and were counted on glass fiber disks, after washing with 5% trichloroacetic acid and ethanol, in a Packard Model 3324 liquid scintillation spectrometer using a toluene scintillator. Standards of ^3H_2O and benzoic-^{14}C acid were used to determine counting efficiency in the dioxane–water–naphthalene–PPO–POPOP homogeneous scintillator system (Langham *et al.*, 1956).

Reaction hypochromicity kinetic data at 260 mμ were obtained from aliquots which were 21-fold aqueous dilutions of the reaction mixture. The average hypochromicity for complete utilization of dATP was 32%. The data for instantaneous incorporation of dAMP were calculated as the ratios of the hypochromicities to 32%. The incorporation data were readily fitted to a straight line, especially in the 10–70% completion range of dAMP incorporation. The slope of each such line was used as k_0, the pseudo-zero-order rate constant in units of nmoles of dAMP incorporated per minute per milliliter.

Protein was removed from completed reactions by heat denaturation and centrifugation, and the supernatants were chromatographically separated into polymer and low molecular weight fractions on a Bio-Gel P-60 column (3.4 × 40 cm, 0.047 M triethylammonium bicarbonate, 1 ml/min). The polymer fraction was then evaporated to dryness, with accompanying volatilization of the triethylammonium bicarbonate, and the residue was taken up in water. Ultraviolet spectrophotometry, liquid scintillation counting, and phosphorus analysis were then carried out (see below).

[2] From Pentex, Inc., Kankakee, Ill., shipped and stored frozen in Dry Ice.

Ten purified polymers, made with dATP, with lengths ranging from 50 to 300 nucleotide units gave an average aqueous $\epsilon(P)_{260} = 9.01 \pm 0.06$. Calculation of the per cent incorporation, Y, of dAMP units was made by dividing the A_{260} units of a quantitatively isolated polymer by 9.01 and then dividing 100 times this result by the number of micromoles of dATP present at the start of the reaction.

In reactions where the initiator was labeled and the dATP was not, the average number, $\bar{n}$, of mononucleotide units in the polymer was calculated by dividing the microcuries per micromole of initiator by the microcuries per monomicromole of resulting polymer. The per cent incorporation, E, of initiator was determined by dividing 100 times the total microcuries in the polymer by the total microcuries in the initiator. If a polymer has the general structure $i_j a_{\bar{x}}$, where i is a monomer unit of the initiator and a is a dAMP unit, $j + \bar{x} = \bar{n}$, and $\bar{x} \cdot E/Y = \bar{x}_{\text{theory}}$, the predicted $\bar{x}$ from the reaction stoichiometry assuming complete utilization of both initiator and dATP.

Experiments and Results

A Test for Nuclease Action in Addase. The polymer d(pT-^{3}H)$_6$(pA)$_{\overline{135}}$ (0.35 mono-μmole) was incubated for 4 hr with 500 units of addase in a total volume of 0.3 ml. After fractionation on P-60, 0.36 mono-μmole of polymer was recovered with no loss of radioactivity and with a structure calculated to be d(pT-^{3}H)$_6$(pA)$_{\overline{136}}$, indicating no detectable 5′-exonuclease and little, if any, endonuclease activities in the addase which would interfere with our use of the enzyme.

Effect of Addase Concentration on Incorporation of d(pT)$_3$. A series of seven 4-hr reactions with d(pT-2-^{14}C)$_3$, 2.0×10^5 dpm/μmole, in which the addase concentration was varied from 250 to 3280 units/ml, gave the per cent incorporation data for d(pT)$_3$ and dATP and the values of $\bar{x}$ for the products d(pT)$_3$-(pA)$_{\bar{x}}$ plotted in Figure 1. The addase specific activity was 1.5×10^4 units/mg. The set of $\bar{x}_{\text{theory}}$ values which resulted from computing the expression $\bar{x}E/Y$ for each reaction showed a very narrow distribution with a value of 100 and standard deviation for a single determination of 1.3%. Thus, the various analytical procedures to determine $\bar{x}$, E, and Y were operating satisfactorily in recovery, accuracy, and precision.

Attempts to Further Purify d(pT-2-^{14}C)$_3$. In order to determine whether the 50% inactivity of the trimer was due to contamination with 3′-hydroxyl-blocked derivative, a sample of d(pT-2-^{14}C)$_3$ was treated at room temperature with concentrated ammonia for 4 hr. The recovered nucleotide material was used as initiator in an addase reaction at 655 addase units/ml, giving a polymer with $\bar{x} = 195$, $E = 50$, and $Y = 97$, indicating no liberation of 3′-hydroxyl function. Paper chromatographic techniques did not reveal the presence of a contaminant.

Effects of Phosphate Concentration and Temperature on Incorporation of d(pT)$_3$. Reactions I–VII (Tables I and II, Figure 2) all contained d(pT-^{3}H)$_3$, $5.20 \times$

TABLE I: Details of First Reactions with Varying Phosphate Concentration and Temperature.

Reaction[a]	Phosphate (M)	Temp (°C)	k_0 (nmoles/min ml)	E (%)	$\bar{x}$[b]
I	0.05	35	7.91	35	310
II	0.14	35	1.38	50	180[c]
III	0.14	15	0.43	75	145

[a] Run at 900 addase units/ml. [b] Unless otherwise noted, $Y = 100\%$. [c] $Y = 83\%$.

TABLE II: Effect of Base, Length, and Conditions on Incorporation of Oligomers.

Reaction[a]	Initiator	Phosphate (M)	Temp (°C)	E[b] (%)	k_0 (nmoles of dAMP incorp/min ml)
VIII	$d(pA)_3$	0.12	15	94	2.13
VI	$d(pT)_3$	0.12	15	74	1.50
IX	$d(pC)_3$	0.12	15	49	1.33
X	$d(pA)_3$	0.04	35	55	—
XI	$d(pT)_3$	0.04	35	32	—
XII	$d(pC)_3$	0.04	35	14	—
XIII	$d(pA)_2$	0.12	15	22[c]	—
XIV	$d(pT)_2$	0.12	15	4.6[d]	—
XV	$d(pC)_4$	0.12	15	91	—

[a] Run at 2600 addase units/ml. [b] Unless otherwise noted, $Y = 100\%$. [c] $Y = 10\%$, $\bar{x} = 200$. [d] $Y = 67\%$, $\bar{x} = 304$.

10^6 dpm/μmole. The addase specific activity was 7×10^3 units/mg. Reactions I–III were the first efforts to evaluate the influence on E of phosphate molarity and temperature changes. Reaction I was a control. Reaction II, with a threefold increase in phosphate molarity, gave an improvement in E from 35 to 50%. Holding at this molarity and dropping the temperature to 15° in reaction III produced another gain up to $E = 75\%$. Concomitantly, both effects lessened the reaction rate, k_0. The addase concentration was then increased threefold, and reactions IVa, IVb, and V–VII (Figure 2 and Table II) were run at 15° with phosphate molarities varying from 0.01 to 0.24 M. Glycerol was added in reactions IVa (0.08 M) and IVb (0.3 M) to prevent denaturation of the addase and to test whether a nonionic solute might effectively replace phosphate in raising E and lowering k_0. From 0.01 to 0.12 M phosphate (Figure 2) E increased, but at 0.24 M very slight improvement over 0.12 M was noted. For further work it was considered that 0.12 M was a practical concentration of phosphate for good incorporation of initiator. The rate-inhibiting effect of increasing phosphate concentration is shown in Figure 2. Glycerol had little or no effect on E or k_0, but another reaction with 0.01 M potassium phosphate and 0.2 M potassium chloride gave $E = 62\%$, showing that chloride can substitute for phosphate.

Effect of Base Composition on Incorporation of Small Oligodeoxy-5'-nucleotides. A comparison of dpA dpC, and dpT as nucleotide units in the initiator was made using $d(pA\text{-}^3H)_3$, 7.73×10^6 dpm/μmole, $d(pC\text{-}^3H)_3$, 1.46×10^7 dpm/μmole, and $d(pT\text{-}^3H)_3$, 5.20×10^6 dpm/μmole. Both 0.12 M phosphate at 15° (reactions VI, VIII, and IX) and 0.04 M phosphate at 35° (reactions X–XII) were used with 2600 units/ml of 7×10^3 units/mg addase; each reaction proceeded until hypochromicity data indicated complete utilization of dATP. The results are shown in Table II (reactions VI and VIII–XII). Under both sets of conditions, $d(pA)_3$ was more efficient than $d(pT)_3$ which, in turn, was more efficient than $d(pC)_3$. Relating this simply to the base in the trimers, the order A > T > C held for E. Furthermore, this order applied for k_0 values under the 0.12 M phosphate at 15° conditions.

Effect of Oligomer Length on Incorporation of Small Oligo-5'-nucleotides. Since it proved to be for all of the trimers that the high molarity–low temperature combination markedly enhanced incorporation, these conditions were extended to the dimers: $d(pA\text{-}^3H)_2$, 5.15×10^6 dpm/μmole, and $d(pT\text{-}^3H)_2$, 3.47×10^6 dpm/μmole. These reactions (XIII and XIV) were both stopped at 31 hr. Reaction XIII gave a 67% yield of polymer with $E = 22\%$ and $\bar{x} = 304$, while reaction XIV produced a polymer with $Y = 10\%$, $E = 4.6\%$, and $\bar{x} = 200$. The presence of radioactivity in the polymer from reaction XIV indicated that $d(pT)_2$ was

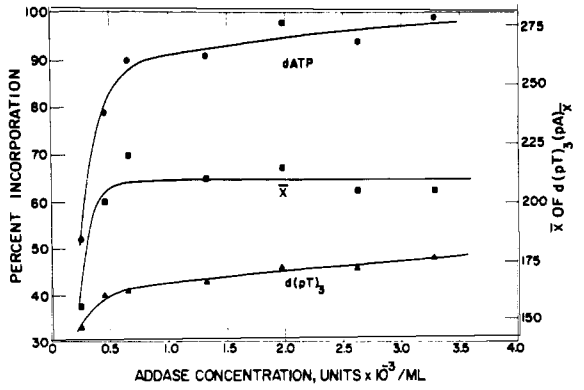

FIGURE 1: Influence of addase concentration on properties of the polymer product from reaction with 10 μM d(pT-2-^{14}C)$_3$ and 1.0 mM dATP (see text for details).

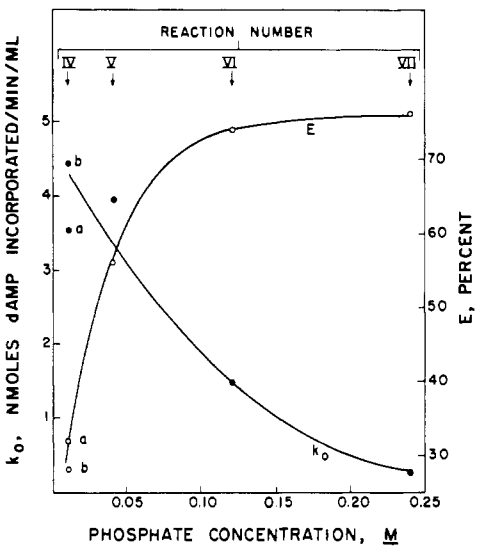

FIGURE 2: Effect of potassium phosphate concentration on E and k_0 for reactions IVa, IVb, and V–VII run at 2600 addase units/ml.

present. Rechromatography on Bio-Gel P-300 in the presence of urea showed no change in specific radioactivity, indicating that the d(pT)$_2$ was firmly bound to the polymer and that this reaction was most likely a genuine case of initiation by d(pT)$_2$.

The tetramer, d(pC-^{3}H)$_4$, 1.95 $\times$ 10^7 dpm/μmole, was used to further test the effect of oligomer length since the corresponding trimer, d(pC)$_3$, gave the poorest incorporation. The result (reaction XV, Table II) was to increase from E = 49% for d(pC)$_3$ to E = 91% for d(pC)$_4$. These three cases not only establish the strong influence of oligomer length on E, but also show that there need not be a sharp transition from no initiator capability for the dimer to competency for the trimer.

Discussion

Bollum (1960, 1962) has developed a method for synthesizing single-stranded polydeoxyribonucleotides with a great variety of base sequences possible toward the 5′ end by virtue of that portion of the polymer coming from a preformed oligodeoxyribonucleotide initiator in the addase reaction. The method fails with a dimer initiator at 0.04 M phosphate and 35° for 90 min (Bollum *et al.*, 1964) but operates with trimer or higher oligomers.

We have synthesized some radioactive initiators in order to follow with needed precision their incorporation in addase reactions which make polymers only a small portion (1–3%) of which is initiator. This report deals mostly with the trimer, since it is the most likely to be used with addase reactions to gain access to polydeoxyribonucleotides containing all possible triplet sequences.

After determining that the addase was free of nucleolytic activity, d(pT-2-^{14}C)$_3$ was used as initiator under the standard conditions, varying addase concentration over a 13-fold range. It was found that about one-half of this initiator could not be incorporated into the polymer even under equilibrium conditions, highly favoring binding of the initiator to the enzyme (Figure 1). The first suspected cause of the trimer impotency was the trimer itself, but attempts to rid it of a possible inactive or inhibiting contaminant were completely unsuccessful.

Both increasing the phosphate concentration and lowering the temperature effected a sharp increase in E. Reactions I and XI, although at the same temperature and concentration conditions as in Figure 1, gave values of E falling significantly below the initiator incorporation curve of Figure 1. This is apparently due to the use of low specific activity addase in the later work. Reactions IV–VII (Figure 2) clearly substantiate the effect of increasing phosphate molarity to give greater E and smaller k_0, while showing again that a sizable increase in addase concentration may have only a slight influence on E.

Experiments with ^{3}H-labeled d(pA)$_3$, d(pT)$_3$, and d(pC)$_3$ were run to compare the new 0.12 M phosphate at 15° with the old 0.04 M phosphate at 35° conditions (Table II, reactions VI and VIII–XII). Again, high molarity and low temperature were beneficial, but a base-specific effect appeared in the order A > T > C in E. Likewise, the base influenced the rate of dAMP incorporation, k_0, in the same order for the trimers. Using ^{3}H-labeled d(pA)$_2$, d(pT)$_2$, and d(pC)$_4$ (reactions XIII–XV), it was shown that dimers can serve as initiators and that oligomer size strongly influences E over the transition range of size from no initiator action to complete utilization.

From the evidence presented it is postulated that, in addition to degree of binding of initiator to addase, competency of binding is an important factor in determining the extent of initiator incorporation into poly-

mer. More than one configuration of the initiator–addase complex may be formed, only one of which is competent to initiate. The enzyme conformation for proper binding should be favored by high phosphate molarity, low temperature, and high addase specific activity. Other considerations affecting competency are the length and base composition of the initiator.

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Nucleoside Phosphonic Acids. I. The Synthesis of 5′-Deoxyuridine 5′-Phosphonic Acids and Derivatives*

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ABSTRACT: The chemical syntheses of the uridylic acid analogs, 5′-deoxyuridine 5′-phosphonic acid (I), 5′-deoxyuridine 5′-phosphonylphosphate (II), and 5′-deoxyuridine 5′-phosphonylpyrophosphate (III), are described. These substances are analogs corresponding to the mono-, di-, and triphosphates of uridine. 5′-Deoxyuridine 5′-phosphonic acid was prepared *via* a Michaelis–Arbuzov-type reaction on 5′-iodo-2′,3′-*O*-isopropylideneuridine with triallyl phosphite. The product of this reaction, diallyl 5′-deoxy-2′,3′-*O*-isopropylideneuridine 5′-phosphonate (IV), was isolated by silicic acid chromatography. Removal of the isopropylidene group on IV was accomplished by treatment with hot 80% aqueous acetic acid, and essentially pure diallyl 5′-deoxyuridine 5′-phosphonate (V) (28%) was isolated after paper chromatography. Catalytic hydrogenolysis of V yielded a mixture of products, 5′-deoxyuridine 5′-phosphonic acid (I) (35%), *n*-propyl 5′-deoxyuridine 5′-phosphonate (50%), and di-*n*-propyl 5′-deoxyuridine 5′-phosphonate (15%), which were separated and isolated by ion-exchange chromatography. The uridine pyrophosphate analogs II and III were prepared by treating inorganic phosphate and pyrophosphate, respectively, with the imidazolidate of I which was prepared by treating I with 1,1′-carbonyldiimidazole. Preliminary attempts to polymerize 5′-deoxyuridine 5′-phosphonylphosphate using polynucleotide phosphorylase were unsuccessful.

A variety of nucleoside analogs have been prepared and their usefulness in biochemical and chemical experimentation is well documented (Bessman *et al.*, 1958; Kahan and Hurwitz, 1962). In general, these analogs have differed from the parent nucleoside or nucleotide by being altered either in the purine or pyrimidine nucleus and/or the carbohydrate portion of the nucleoside. Recently analogs of nucleotides have been described which have a phosphite (Hall *et al.*, 1957; Holy *et al.*, 1965) and phosphonate (Parikh *et al.*, 1957; Wolff and Burger, 1959; Bannister and Kagan, 1960) group in place of the naturally occurring phosphate group. The synthesis of a nucleoside triphosphate analog has been reported (Myers *et al.*, 1963) which contains a methylene group in place of the terminal pyrophosphate oxygen. This analog, 5′-adenyl methylenediphosphonate, is capable of being enzymatically polymerized by the deoxyribonucleic acid- (DNA) directed ribonucleic acid (RNA) polymerase of *Escherichia coli* (Simon *et al.*, 1965). Presently very little is known of the chemistry and biochemistry of nucleoside phosphonic acids, the analogs of nucleo-

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