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POLYDEOXYADENYLIC ACID

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A reaction resulting in the addition of deoxynucleotidyl residues to the 3′-hydroxyl group of oligodeoxynucleotides has been observed in partially purified preparations of calf thymus DNA polymerase.[1, 2] The reaction was first thought to be a manifestation of the DNA polymerase, but subsequent investigation has demonstrated a *terminal*-deoxynucleotidyl transferase that is distinct and separable from DNA polymerase (*replicative*-deoxynucleotidyl transferase). This distinction was first recognized by Krakow *et al.*[3] in their work on polymerizing enzymes in extracts of calf thymus nuclei, and somewhat later by Keir and Smith[4] and by us.[5] We have now succeeded in preparing the terminal-addition enzyme[6] free of polynucleotides, deoxypolynucleotide degrading enzymes, and DNA polymerase, and find that it displays a marked, although not absolute, preference for d-ATP polymerization.

When the deoxyadenylate is grafted on to oligodeoxynucleotide initiators of known sequence and structure, sequence and structure is known throughout the entire length of the new graft copolymer. The fact that polymerization takes place on a fixed number of growing points allows us to predict that molecular weight distributions will be rather narrow, and it is possible to control the average degree of polymerization by limiting the amount of monomer. Thus, a large series of polydeoxynucleotides having definite sequence, structure, and chain length may be prepared. Some of the polymers available from the action of this enzyme are of special interest to current work on information transfer in biological systems, and they form the principal subject of this communication.

Materials and Methods.—Definitions: The term "initiator" is here defined as a polymer of deoxynucleotides having a free 3′-OH that will accept the deoxynucleotidyl residue from deoxy-

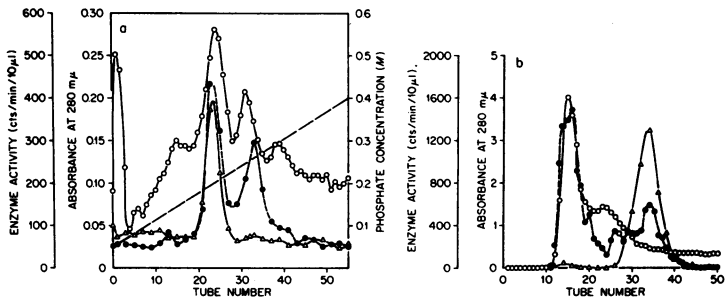

FIG. 1.—Separation of deoxynucleotidyl transferases. (*a*) Chromatography on hydroxylapatite (1 × 14 cm) at pH 7.5. (*b*) Gel filtration on Sephadex-G-100 (3 × 80 cm) at pH 7.2, 0.05 *M* phosphate. The sample shown here had not been chromatographed on hydroxylapatite. ○ = A^{280}; ● = DNA polymerase; △ = terminal-addition enzyme.

nucleoside triphosphates. The term "template" is used to describe similar polymers that have a function in determining base sequence by complementary H-bonding. When a polymer is greater than 90% deoxyadenylate, "polydeoxyadenylate" is used as a general descriptive term. If the initiator portion is large compared with the polyadenylate region we call them "modified polymers" (e.g., "modified DNA"). The final products are in all cases the equivalent of linear graft copolymers.

Substrates: Deoxyribonucleoside triphosphates and diphosphates were obtained from commercial sources. P^{32}-deoxyribonucleoside triphosphates were prepared as previously described.[7] Oligodeoxythymidylates, dT_2 to dT_9, were synthesized and purified according to Khorana and Vizsolyi.[8] Oligodeoxyadenylates, dA_2 to dA_{11}, were prepared by DNase I degradation of polydeoxyadenylate (cf. Fig. 3). Heterogeneous oligodeoxynucleotide, dX_{3-8}, was prepared by DNase I degradation of calf liver DNA and partially purified by chromatography on Dowex-1-acetate (cf. ref. 9). Calf thymus DNA was purchased from Worthington Biochemical Corp., Freehold, N. J. It was dissolved in 0.02 *M* Tris-Cl, pH 8.1, and heat-denatured before use.

Enzymes and enzyme assays: DNase I, yeast inorganic pyrophosphatase, and venom phosphodiesterase were obtained from Worthington Biochemical Corp.

Incorporation assays for DNA polymerase contained: the deoxyribonucleoside triphosphates of adenine, guanine, cytosine, and thymine, one of which was labeled with C^{14} or P^{32}; denatured DNA; Mg^{2+}; mercaptoethanol; enzyme; and phosphate buffer, pH 7.0 as in previous work.[7] Aliquots of reaction mixtures were placed on filter paper disks and worked up as described ealier.[10] Routine incorporation assays for terminal-addition enzyme contained: a single C^{14}- or P^{32}-labeled deoxyribonucleoside triphosphate, 0.1 mM; 0.01 mM $d(pT_6)$; 8 mM Mg^{2+}; 1 mM mercaptoethanol; enzyme; and 40 mM phosphate buffer, pH 7.0. Aliquots of reaction mixtures were placed on filter paper disks and worked up as above.[11]

Spectrophotometric assays for terminal-addition polymerization contained 1 mM dATP; 0.01 mM oligodeoxynucleotide; 8 mM Mg^{2+}; 1 mM mercaptoethanol; 40 mM potassium phosphate buffer, pH 7.0; and terminal addition enzyme. Aliquots (usually 50 μl) were taken from the reaction mixtures and diluted with 3.0 ml of 0.15 *M* NaCl containing 1.0 mM Versene and 50 mM Na acetate buffer, pH 4.8. Absorbancy of the diluted aliquot was measured at 260 mμ. An optical density decrease of 6.0 corresponds to polymerization of 1 μmole of dATP.

Preparation of terminal-addition enzyme: A partially purified preparation of calf thymus DNA polymerase (roughly equivalent to fraction D of an earlier preparation[7]), chromatographed on a hydroxylapatite column, yields two activity peaks when assayed with all four deoxyribonucleoside triphosphates and DNA primer (Fig. 1*a*). Only peak 1 responds to oligodeoxyribonucleotide initiators and single deoxynucleoside triphosphates. Peak 2 requires all four deoxynucleotides and high molecular weight DNA (or deoxypolynucleotide) template for activity. Gel filtration of peak 1 on Sephadex G-100 (cf. Fig. 1*b*) produces a peak at 1 column volume corresponding to inactive protein and any contaminating DNA polymerase. A second protein peak at 1.7 column volumes contains all the terminal-addition enzyme. The active peak from G-100 is concen-

trated by $(NH_4)_2SO_4$ precipitation and is stable at $-20°C$ for long periods. It has a specific activity of about 15 μmoles dATP/hr/mg protein. The details of this purification procedure will be published separately.[6]

Polymer purification and analysis: The terminal-addition reaction mixtures were made 0.5 *M* in NaCl, and the polydeoxyadenylates were precipitated by addition of two volumes of 95% ethanol. This procedure was repeated several times, and the product was then clarified by centrifugation to remove any turbidity from denatured protein. Residual deoxyribonucleoside triphosphate and orthophosphate were removed by dialysis against 0.2 *M* NaCl, and the polymer was reprecipitated with ethanol.

The base compositions of polymers and modified DNA were determined after hydrolysis in 98% formic acid in sealed tubes. The hydrolysate was chromatographed on Whatman #1 in isopropanol:HCl:H_2O (68:16:16), and the bases were determined by spectrophotometric analysis of ultraviolet absorbing areas after extraction with 0.1 *N* HCl.

Sedimentation rates were determined on solutions of polymers having an A^{260} of about 0.5, in 0.15 *M* NaCl containing 0.015 *M* Na_3 citrate, at 20°C in the AN-D rotor of the Spinco Model E centrifuge. The UV absorption photographs were traced with a Joyce-Loebel recording microdensitometer.

Results.—Properties of the terminal-addition enzyme: (a) *Specificity for dATP polymerization:* Separate substrate saturation curves for dATP, dGTP, dCTP, and dTTP indicated that the rate of dATP polymerization on pT_6 increases up to about 1–2 mM dATP and then decreases. In contrast, no further increase beyond the rate of 0.1 mM is found for dCTP, dGTP, or dTTP under the conditions described. A maximum specific activity of 15 μmoles dATP/hr/mg protein is observed while dCTP, dGTP, or dTTP polymerize at less than $^1/_{50}$ that rate. Similar results were obtained in both incorporation and optical assays.

(b) *Chain length requirement for initiator:* Figure 2 demonstrates use of the optical assay to test various oligodeoxynucleotide initiators. As shown previously,[2] dinucleotides are ineffective while oligodeoxynucleotides with chain-length 3 or greater act as initiators. Other experiments confirmed the earlier findings[2] that the reaction is specific for deoxyribonucleoside triphosphates and oligodeoxynucleotide initiators. That is, *ribo*nucleoside triphosphates are not polymerized on oligo*ribo*nucleotides or oligodeoxyribonucleotides, deoxyribonucleoside triphosphates are not polymerized on oligo*ribo*nucleotides, and deoxyribonucleoside diphosphates and *ribo*nucleoside diphosphates are not polymerized under the conditions employed.

(c) *Other properties:* Initiator concentration curves show an optimum dATP polymerization rate at 0.01 mM pT_6 (110 μg enzyme protein/ml) and an inhibitory effect at higher initiator concentrations. The optimum pH for dATP polymerization is at 6.7–7.0 in phosphate buffers. Use of barbital buffer resulted in complete inhibition. Pyrophosphate inhibition was 33 per cent at 1 mM and 100 per cent at 10 mM.

Preparation of polydeoxyadenylates and oligodeoxyadenylates: (a) *Synthesis of* $d(X_mA_n)$: A 250-ml reaction mixture containing: 8 mM MgCl; 40 mM potassium phosphate, pH 7.0; 1 mM mercaptoethanol; 1 mM dATP; 37 μg crystalline-

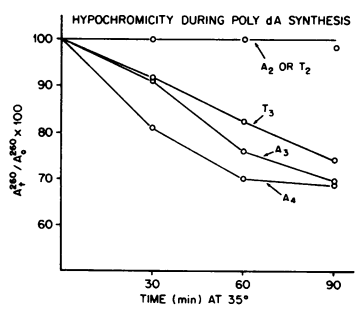

FIG. 2.—Optical assay for terminal deoxynucleotidyl transferase. (See text for details.) A_t^{260}/A_0^{260} is the ratio of absorbancy at time, *t*, to that at zero time.

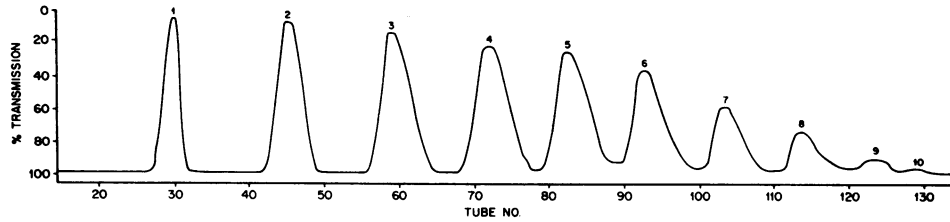

FIG. 3.—Chromatography of DNase I digest of polydeoxyadenylate. A linear gradient of 0.02 *M* Na acetate, pH 4.8 (1 l.) to 0.4 *M* NaCl, 0.02 *M* Na acetate, pH 4.8 (1 l.) was used to elute a 1 × 25-cm DEAE column.

yeast pyrophosphatase; 180 A^{260} units of DNase I oligodeoxyribonucleotide (dX_{3-8}); and 10 mg terminal-addition enzyme was incubated for 3 hr at 35°C. At this time, the A^{260} of a diluted aliquot was about 65 per cent of the initial reading. A second portion of dATP (300 μmoles) was added and the incubation continued for 5 hr. A diluted aliquot again indicated complete polymerization of monomer, and a third portion of dATP (300 μmoles) was added. The incubation was continued overnight. After purification and dialysis as described above, the final yield was about 6000 A^{260} units, or roughly 0.23 gm.

(b) *DNase I degradation of* $d(X_mA_n)$*:* One thousand A^{260} units of the product synthesized above was digested 16 hr with 5 mg of DNase I in the presence of 0.01 *M* $MgCl_2$. Chromatography of this digest on DEAE produced the pattern shown in Figure 3. Peaks 1 through 10 all had adenylic acid spectra, and analyses for total and terminal phosphate gave ratios of approximately 2, 3, 4, etc., respectively. The material isolated in peak 1 does not initiate end addition, whereas peaks 2 through 10 do. Since oligodeoxynucleotides of tri- or higher complexity are required for initiation[2] (cf. Fig. 2), the respective peaks in Figure 3 are the homologous series of di- to undecanucleotides of deoxyadenylate.

(c) *Synthesis of homogeneous poly dA:* Twelve and one-half A^{260} units of peak 2 [trinucleotide, d(pApApA)] were incubated with 200 μmoles of dATP and 1 mg of terminal-addition enzyme. The product, 1200 A^{260} units, was isolated as described above and is defined as homogeneous poly dA by its mode of preparation, greater than 99 per cent of its total nucleotide content arising from polymerization of dATP. Trace contaminants remaining after initiator chromatography (Fig. 3) or present in the substrate used cannot be excluded by the type of analyses detailed here.

(d) *Synthesis of other polydeoxyadenylates,* $d(T_mA_n)$*, and modified DNA (DNA-*A_n*):* Reaction mixtures of 10-ml volume containing a suitable initiator (0.1 μmole oligo dT_3–dT_9 or 10 μmoles DNA-P), 1 mM dATP, and 400 μg terminal-addition enzyme provide polymers with n = 100 or 300, depending upon the amount of monomer allowed to polymerize. One hundred to 250 A^{260} units of each polymer were obtained after purification by ethanol precipitation and dialysis as described above. No residual oligo dT could be detected by chromatography of these polymers on DEAE paper.[10] Base analysis of the modified DNA indicated that about 300 dAMP residues were added per DNA chain of MW 3×10^6 (cf. ref. 3).

Analysis of polydeoxyadenylates: (a) *Spectral properties:* The analytical data for the $d(T_mA_n)$ series of polymers are shown in Table 1. The absorbancy at 260

mμ per μmole of phosphate, E(P), averages 8.7 when absorbancy is measured at neutral pH in distilled water. Formation of what may be presumed to be a helical form of polydeoxyadenylate[12] is indicated by the reduced absorbancy and change in λ_{max} at pH 4.0 (Fig. 4).

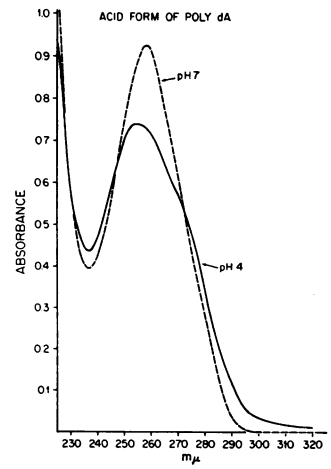

FIG. 4.—Spectrum of homopoly dA at neutral and acid pH.

(b) *Base analysis:* The data for the chain lengths listed in Table 1 were computed from base analyses. Since the polydeoxyadenylates contain only 1–10 mole per cent thymine, rather large samples (about 30 A^{260} units) are required for analysis. In the higher series, thymine is near the limit of detection. Thus while some of the analyses are not precise, they do define a *lower limit* of chain length.

(c) *Sedimentation analysis:* Each of the polydeoxyadenylates sediments with a single boundary and the sedimentation constants ($S_{20,w}$) are in the range 2.2–4.7 (Table 1). The shape of the sedimenting boundary indicates a rather narrow distribution of molecular weights. Our sedimentation studies and end-group analyses are in remarkably good agreement with the sedimentation and viscosity results of Fresco and Doty[13] on poly*ribo*adenylate. More detailed sedimentation studies are required for greater precision and for analysis of molecular weight distributions. The results from the analytical ultracentrifuge are therefore to be taken as a qualitative comparison that indicates the polymeric nature of the polydeoxyadenylates and demonstrates that polymers of varying sedimentation rates can be prepared.

(d) *Biological properties:* The homopolymer of deoxyadenylate serves as a template for synthesis of poly*ribo*uridylate by the DNA-dependent RNA polymerase from *Azotobacter vinelandii*.[14] The polydeoxyadenylates and modified calf thymus DNA are, by themselves, poor templates for calf thymus DNA polymerase. Addition of oligothymidylate with chain length greater than 5 as an initiator permits use of the polydeoxyadenylates and modified DNA as templates for complementary syntheses. Oligodeoxyadenylates or heterogeneous oligodeoxynucleotide will not initiate synthesis.[15]

Discussion.—Evidence for the polymeric nature of the polydeoxyadenylates may be summarized as follows: A loss in absorbancy is noted during synthesis,

TABLE 1

ANALYSIS OF POLYDEOXYADENYLATES

Description	n = 100 (theoretical)* E(P)	n(actual)*	$S_{20,w}$	n = 300 (theoretical)* E(P)	n(actual)*	$S_{20,w}$
$d(T_3A_n)$	9.27	180	3.70	8.01	283	4.69
$d(T_4A_n)$	8.37	135	3.15	8.73	252	4.43
$d(T_5A_n)$	8.98	102	2.31	8.04	258	4.08
$d(T_6A_n)$	8.22	85	2.18	8.52	308	4.15
$d(T_7A_n)$	8.61	109	2.28	7.98	376	4.12
$d(T_8A_n)$	9.50	70	2.18	9.05	362	4.06
$d(T_9A_n)$	9.00	99	2.37	9.63	296	3.89
Average	8.84	111	2.59	8.56	305	4.20

* n (actual) determined by base analysis; n (theoretical) estimated from the polymerization recipe, i.e., $n = \frac{\mu\text{moles monomer}}{\mu\text{moles initiator}}$.

and the isolated product is hypochromic with respect to its constituent nucleotides. The products formed are insoluble in 0.6 *N* $HClO_4$ and 66 per cent ethanol:0.3 *M* NaCl. At acid pH, a further hypochromicity and change in λ_{max} suggests formation of helical poly dA. Upon degradation to mononucleotides by venom phosphodiesterase A^{260} increases 50 per cent. Degradation of the polymers with DNase I produces a homologous series of oligodeoxyadenylates of chain length 2 to 11. Each of the many polymers prepared sediments as a single species, and the approximate sedimentation rate can be predicted from the polymerization recipe.

The outstanding feature of the enzymic polymerization of a given monomer on an initiator of known sequence is the formation of a known, mixed sequence at the 5′-phosphoryl end of the product molecule. Even the simple copolymers described here (Table 1) have several types of coding units, e.g., TTTAAA..., TTTTAA..., TTTTTA..., etc. Recent developments in the stepwise synthesis of oligodeoxynucleotides of tri- and higher complexity,[16, 17] and isolations from DNase I digests[9, 18] or from diesterase digests of UV-irradiated DNA[19] provide a number of initiators that may now be incorporated into polymers of known sequence. The known sequence in a definite location can then be used for the synthesis of complementary deoxypolynucleotides by DNA polymerase, or of complementary ribopolynucleotides by RNA polymerase. Since the polydeoxyadenylates can act as templates for DNA polymerase, forming complementary double-stranded products,[15] the specific sequence and its complement can be tested in single- or double-stranded form. Thus, while the synthesis of -pApA- sequences as the primary action of the terminal-addition enzyme seems somewhat monotonous, incorporation of the initiator sequence at a known point in a high polymer provides a useful point of reference. The location, size, and nature of the sequence are landmarks in the polymers that may aid in assessing polarity and coding ratios in polypeptide synthesis.[20]

Preliminary tests of biological activity of the polydeoxyadenylates in several enzyme systems indicate that they are active. They should therefore allow some rather detailed model experiments related to information transfer in these biological systems.

Summary.—Known sequences of three or more deoxynucleotides can be incorporated onto the phosphoryl end of polydeoxyadenylate polymers by virtue of the fact that oligodeoxynucleotides are required to initiate polymerization of dATP catalyzed by a *terminal* deoxynucleotidyl transferase from calf thymus gland. Chain length of the polydeoxyadenylate produced can be controlled, and molecular weight distributions are expected to be narrow. The biosynthetic polydeoxyadenylates provide simple copolymers of defined sequence, structure, and chain length for model studies on information transfer in enzyme systems.

Note added in proof: dITP polymerizes enzymatically at about one half the rate of dATP, allowing preparation of useful amounts of polydeoxyinosinates through the action of the *terminal* deoxynucleotidyl transferase. Use of homologous series of oligodeoxynucleotide initiators (e.g., T_m, A_m, C_m, I_m; where m varies from 3 to 9) for dITP and dATP polymerizations therefore generates an appreciably larger number and variety of sequences in this graft copolymer series (e.g., T_m-A_n, T_mI_n, A_mI_n, I_mA_n, C_mA_n, C_mI_n) and in the complementary dA:dT and dI:dC series that may be derived from the graft copolymer series through their use as templates for calf thymus DNA polymerase.

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*LETHALITY AND THE STIMULATION OF RNA SYNTHESIS BY STREPTOMYCIN**

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The reactivity of streptomycin, neomycin, and other related antibiotics with DNA[1, 2] led us to explore the hypothesis that a reaction with DNA in the bacterial chromosome may be the basis of the lethal action of this class of antibiotics. Since the basicity of streptomycin permits ready reaction with most of the cellular anionic components, the numerous inhibitory effects of streptomycin with separated components observed *in vitro* cannot be expected to point clearly to the nature of the primary lethal action within the cell. We have attempted to dissect the problem of the primary lethal action of the drug in intact bacteria, using a variety of nutritionally deficient organisms, particularly *E. coli* strain TAU.[3] Since it had been reported[4] that the chromosome of strain TAU could be manipulated by a nutritional technique, we hoped to relate sensitivity of the organism to the antibiotic to the