ENZYMATIC SYNTHESIS OF DEOXYRIBONUCLEIC ACID, XVI. OLIGONUCLEOTIDES AS TEMPLATES AND THE MECHANISM OF THEIR REPLICATION*

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Communicated December 30, 1963

DNA[1] in the molecular weight range of a million or more serves as a primer for replication by DNA polymerase but the minimal size of a DNA molecule that will function has not been determined. Extensive digestion with pancreatic deoxyribonuclease destroys the priming capacity of a DNA preparation,[2] and the use of partial digests has failed to establish the range and relative effectiveness of molecules of intermediate size. With the availability of synthetic deoxyribopolynu-

cleotides of defined size and structure, the influence of polymer size on priming can now be studied.[3] In particular, the study of the behavior of the synthetic compounds of the type $T(AT)_nA$ as possible primers for replication is of great interest to an understanding of the *de novo* enzymatic synthesis of the deoxyadenylate-deoxythymidylate (dAT) copolymer.[4, 5] In this report we describe the capacity of certain members of the $T(AT)_nA$ oligomer series to prime the enzymatic synthesis of the dAT copolymer. The replication of these oligomers at different temperatures in the range of 0–45° suggests a possible mechanism of replication which is novel in our experience with the DNA polymerase of *Escherichia coli*.

Materials and Methods.—The following deoxyribo series of oligonucleotides were synthesized by the method of Weimann, Schaller, and Khorana[3] and for ease of reference in this paper are designated by the trivial notation $(AT)_n$:

d-pTpApTpA—$(AT)_2$	d-T(pApT)$_4$pA—$(AT)_5$
d-pT(pApT)$_2$pA—$(AT)_3$	d-T(pApT)$_5$pA—$(AT)_6$
d-pT(pApT)$_3$pA—$(AT)_4$	d-T(pApT)$_6$pA—$(AT)_7$

Melting curves for dAT polymer, determined spectrophotometrically in 0.04 *M* potassium phosphate buffer at pH 7.0, gave a T_m of 56.7°. When the solution was adjusted to 0.004 *M* in $MgCl_2$, the T_m was increased to 61.7. No hyperchromic transitions for $(AT)_{3,4,6}$ (less than a 5% increment) could be obtained in the range of 0.3–62° in 0.04 *M* potassium phosphate buffer, pH 7.0 and 0.004 *M* $MgCl_2$; it may be inferred that helical conformations were absent or in very low concentration. Extinction coefficients at 260 mμ based on phosphate residues were 6,650 for $(AT)_{>1000}$, 9,600 for $(AT)_6$, and 12,600 for $(AT)_3$.

Preparation of the dAT polymer (also referred to as $(AT)_{>1000}$ since estimates of its mean molecular weight are 6×10^6 or greater) and sources of DNA are as previously described.[6] We are grateful to Dr. R. Haselkorn, the late Dr. R. K. Morton, and Dr. C. Schildkraut for gifts of the DNA samples from *Tetrahymena patula* (a protozoan), yeast cytochrome b_2, and *Dictyostelium discoideum* (a slime mold), respectively. Fraction IX (hydroxylapatite) of *E. coli* DNA polymerase[7] was used throughout.

Quantities of oligomers and dAT are referred to in terms of moles of polymer, except where otherwise designated.

The standard incubation mixture contained 20 μmoles of potassium phosphate buffer, pH 7.0, 2 μmoles of $MgCl_2$, 200 mμmoles of 2-mercaptoethanol, 150 mμmoles of dATP and dTTP, and 380 units of polymerase in a total volume of 0.50 ml. Measurements were made in glass-stoppered cuvettes with a 0.2-cm light path in a Zeiss spectrophotometer PMQ II equipped to permit ±1° temperature control over the range of 0–45° by circulating ethylene glycol or water through the jacketed cell housing. The reaction mixtures were equilibrated at each temperature for 30–60 min before the enzyme (0.01 ml) was added. To conserve the supply of oligomers, reactions were also run in a reaction mixture of 0.20 ml containing the components in proportionately reduced quantities. The same cuvettes served when raised by 0.7-cm shims placed in the cuvette holder. To cleanse the cuvettes of the dAT polymer produced, it was necessary to treat them with chromic acid at 50° and 2 *M* NH_4OH.

Results.—Priming of dAT synthesis by a series of $(AT)_n$ oligomers at 37°: In the presence of dATP, dTTP, and DNA polymerase, but in the absence of any added primer, there is no apparent reaction for several hours; a rapid production of a high-molecular weight dAT polymer then takes place.[4, 5] Previous studies have established that during the long lag period of this *de novo* (unprimed) synthesis of dAT a few full-sized molecules are produced which then are replicated with exponential kinetics.[5] Figure 1*a* shows the results of testing a series of oligomers for their capacity to substitute for dAT polymer in priming dAT synthesis. The decrease in absorbancy, a measure of polymer synthesis, occurred promptly when $(AT)_7$, $(AT)_6$, or $(AT)_5$ were used and considerably earlier than the *de novo* reaction

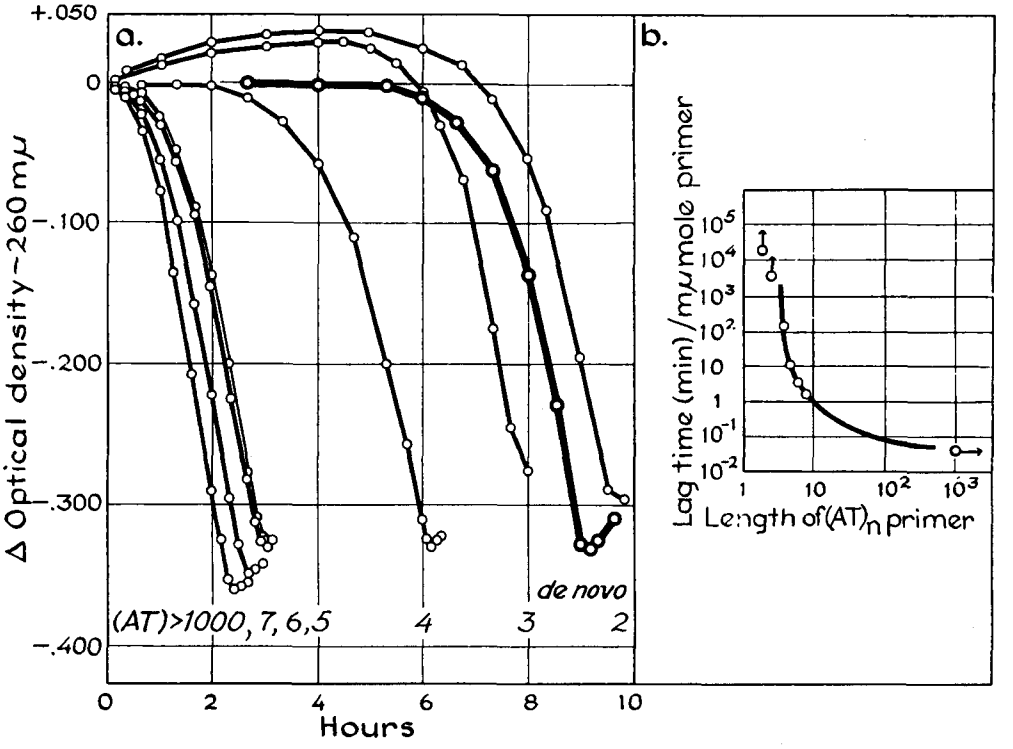

FIG. 1.—Priming of dAT synthesis by $(AT)_n$ oligomers at 37°C. (*a*) The reaction mixtures (see *Materials and Methods*) were primed with 0.002 mμmole of dAT, 0.045 mμmole of $(AT)_7$, 0.075 mμmole of $(AT)_6$, 0.21 mμmole of $(AT)_5$, 0.90 mμmole of $(AT)_4$, 15 mμmoles of $(AT)_3$, or 50 mμmoles of $(AT)_2$, and compared to a typical *de novo* synthesis curve at 37°. (*b*) The length of $(AT)_n$ polymer refers to the number (n) of AT dinucleotide residues in the polymer. The amount of polymer in mμmoles was calculated from the $E_{260m\mu}$ for the polymers (see *Materials and Methods*). The $E_{260m\mu}$ under reaction conditions for $(AT)_{2,4,5,7}$ were assumed to be 12,600, 12,600, 10,000, and 7,000, respectively. The symbols with an arrow attached are undetermined values that are at least greater than as represented on the graph.

when $(AT)_4$ was present. No significant effect on the lag time of the *de novo* reaction could be attributed to the use of $(AT)_3$ or $(AT)_2$. This priming effect of the oligomers $(AT)_{4-7}$ was indistinguishable from that of dAT in requiring the presence of both dATP and dTTP as well as DNA polymerase and Mg^{++}.

The kinetics of dAT synthesis, primed or *de novo*, were previously described as exponential,[5] but semilog plots of the data in Figure 1 or of dAT-primed reactions under present conditions were not straight-line functions. The difference between the present and earlier results is probably due to the absence of endonuclease I[8] in the more purified polymerase preparation in current use. Without scissions by endonuclease, longer molecules are produced, and new priming points are not generated. As a result, polymerase molecules, present in great excess, are not engaged for the synthesis of additional dAT polymers. In support of this interpretation are the results obtained on adding 0.0002–0.02 unit of endonuclease I to oligomer- or dAT-primed reactions. The lag times were reduced, the reaction rates were markedly increased, and the kinetics became nearly exponential.

The kinetics of the oligomer-primed syntheses, the extent of these reactions, and the final destruction of polymer by the exonuclease II component of polymerase[9]

upon exhaustion of the substrates were not distinguishable from the reactions primed by dAT. The product of the $(AT)_{4-7}$-primed reactions is a large molecule as judged by its complete acid-insolubility and nondialyzability. Evidence for its being a dAT copolymer is based on a CsCl density gradient analysis of a product of an $(AT)_4$-primed reaction. A single sharp band at the density of dAT was found both at pH 8 and at pH 12, which is beyond the alkaline-melting transition.[10]

The priming capacities of the $(AT)_n$ series of oligomers and of dAT polymer have been compared on the basis of the lag time per mμmole of primer (Fig. 1*b*). The lag time, arbitrarily defined as the time elapsed when an absorbancy decrease of 0.020 is reached (about 6% of the total reaction), is inversely proportional to the concentration of a given primer. For example, the lag times in an $(AT)_6$-primed reaction was 24, 42, and 83 min, respectively, with additions of 0.18, 0.12, and 0.06 mμmole of primer.[11] The lag time is markedly influenced by the size of the primer (Fig. 1*b*) with dAT being at least 100 times more effective on this basis than the oligomers. It is not clear whether the greater effectiveness of a primer is due to more efficient binding by the enzyme or to its superior capacity to initiate and sustain replication.

Influence of temperature on priming capacities of dAT and of the $(AT)_n$ oligomers: All previous studies on the enzymatic replication of DNA have been carried out at 37°. Under standard reaction conditions this temperature is 25° below the T_m of dAT, and it might be considered that variations between 0 and 45° attributable to the primer would not have a profound effect on dAT replication. However, even within the 0–45° range, there might still be significant changes in the degree of strand separation at the end of the dAT helix and very likely large effects on the secondary structure of the $(AT)_n$ oligomers. As seen in Figure 2, the initial rates of replication are strikingly affected by temperature. With dAT as primer (Fig. 2*a*), the reaction at 20° is far slower than at 37°; at 10° it is barely detectable. By contrast, the reaction with $(AT)_4$ as primer (Fig. 2*e*) is slightly faster at 20° than at 37° and more rapid still at 10°. The replication rates of $(AT)_5$, $(AT)_6$, and $(AT)_7$ over the 10–45° range (Fig. 2*b*, *c*, *d*) show patterns that form a transition between those of $(AT)_4$ and dAT. The lag time of the *de novo* reaction varied from 6–12 hr at 37°; no reaction was detectable during a 24-hr period at 20° or a 72-hr period at 10°; at 45° the lag time was greater than 10 hr.

The fact that the priming by $(AT)_4$ was better at 10° than at higher temperatures encouraged attempts to observe priming by $(AT)_3$ at low temperatures. While no reaction was observed after a 72-hr incubation with $(AT)_3$ at either 10° or 0°, a subsequent elevation of the temperature to 37° resulted in a prompt development of polymer (Fig. 2*f*). Controls which were preincubated for 72–144 hr at 0° without enzyme or controls lacking $(AT)_3$ during the preincubation period showed the usual *de novo* lag period when placed at 37°. These findings suggest that a few larger molecules were produced at 0° which were unable to function effectively until the temperature was raised. A preliminary study of the kinetics of the $(AT)_3$-primed reaction at 0° for 24, 48, and 72 hr showed a progressive decrease in lag time when the temperature was then raised to 37°.

The relative rates at various temperatures for each of the $(AT)_n$ oligomers cited in Figure 2 are compared in Figure 3 on the basis of lag time. The temperatures in the 0–45° range were selected arbitrarily; measurements above 45° (e.g., 50°)

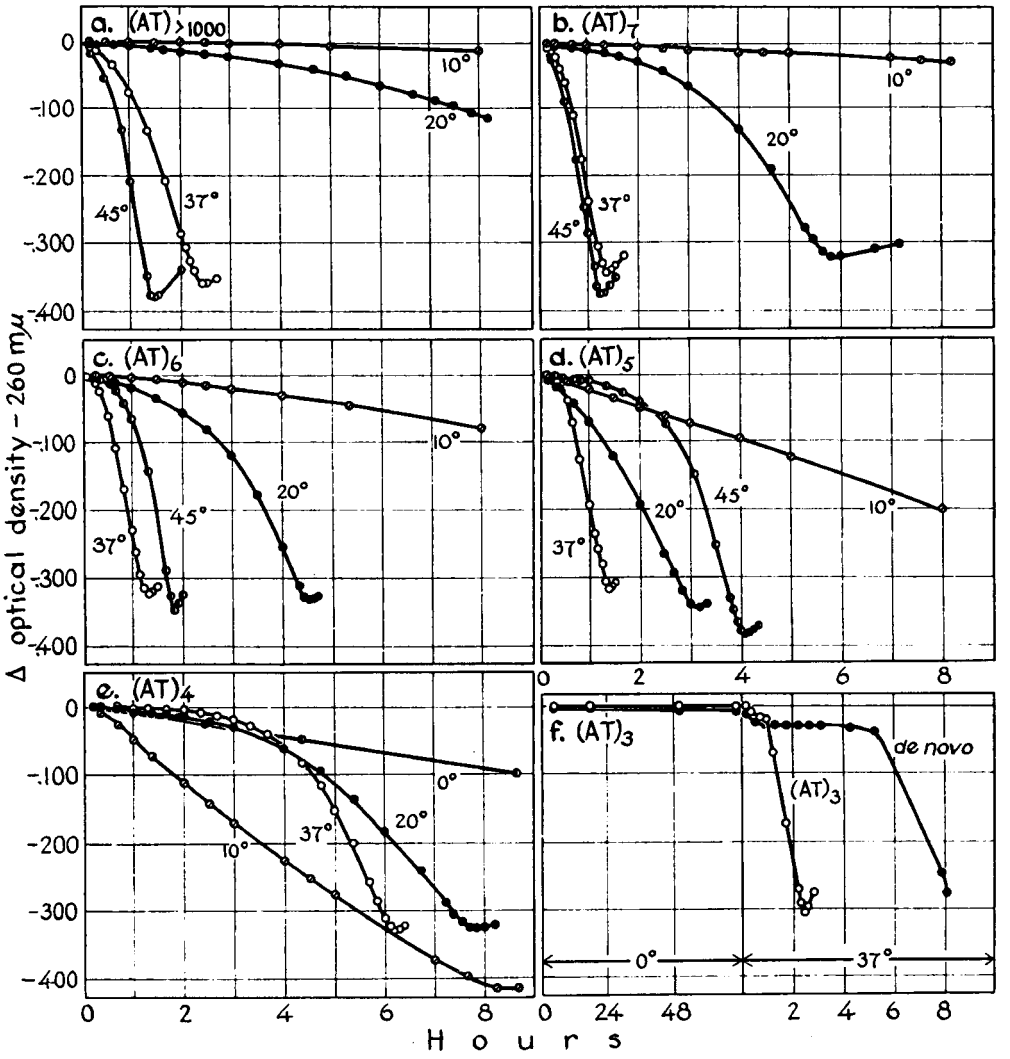

FIG. 2.—Priming of dAT synthesis by $(AT)_n$ oligomers at various temperatures. Reaction mixtures as described were primed at the indicated temperatures with (*a*) 0.002 mμmole of dAT, (*b*) 0.13 mμmole of $(AT)_7$, (*c*) 0.22 mμmole of $(AT)_6$, and (*d*) 0.40 mμmole of $(AT)_5$. In (*e*) curves at 10°, 20°, 37° were obtained by priming with 0.9 mμmole of $(AT)_4$. The curve at 0° was obtained with 0.5 mμmole of $(AT)_4$ and adjusted by comparison with a curve obtained with this level of $(AT)_4$ at 10°. (*f*) Reaction mixtures, one primed with 15 mμmoles of $(AT)_3$ and one without any primer (*de novo*) were incubated at 0° for 72 hr, then followed in the spectrophotometer at 37°.

were complicated by extremely rapid rates for the dAT-primed reaction and by enzyme inactivation. It is clear from Figure 3 that an optimal temperature has been approximated for the replication of each oligomer in the series and that there is a direct correlation between this temperature and the size of the oligomer.

Priming of dAT synthesis by DNA: The capacity of the relatively short AT sequences, as in the $(AT)_n$ oligomers, to prime dAT synthesis suggested the possibility that such sequences in DNA chains might also prime dAT synthesis. The crab testis DNA component, rich in AT copolymeric sequences but containing about 3 per cent guanine and cytosine interspersed through the chains,[6] initiated

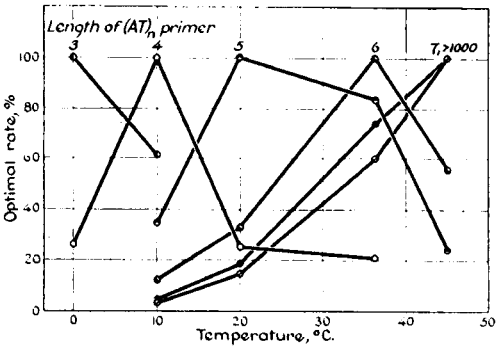

FIG. 3.—Optimal temperatures of priming by $(AT)_n$ oligomers. The optimal rate of priming defined as the minimum time required to produce a decrease of 0.020 in optical density at 260 mμ, was calculated from the data in Fig. 2 and set at 100. The entry for $(AT)_3$ at 10° is based on the lag time at 37°, after a prior incubation for 72 hr at 10° as compared with a sample preincubated at 0°.

the prompt development of polymer (Fig. 4, #2). It may be inferred that the product is a dAT polymer since dGTP and dCTP were excluded from the reaction mixture and the product displayed the buoyant density pattern in alkaline CsCl characteristic of dAT. DNA samples from yeast cytochrome b_2, *T. patula*, and whole yeast also caused significant reductions in lag time (Fig. 4). On the other hand, T2 DNA, relatively rich in AT content (65% AT), failed to prime while phage λ DNA, with a relatively low AT content (51%), appeared to do so.

Discussion.—The synthesis of a dAT polymer in response to an oligomer with an alternating sequence of deoxyadenylate and deoxythymidylate residues indicates that such an oligomer is being used as a template for the *E. coli* DNA polymerase. The lack of a 5′-phosphoryl terminal group in certain of these active oligomers indicates further that such a terminal grouping is not a requirement in the replication process. The kinetics of replication of the oligomers and the synthesis of exclusively large molecules suggest that a few oligomers have primed the synthesis of a few macromolecules which are then more rapidly replicated. These observations support an earlier hypothesis[5] that the *de novo* synthesis of dAT polymer starts with a random polymerization to produce a rare $(AT)_n$ oligomer. Just how this rare oligomer is put together in the first place remains unknown, but the fact that such an oligomer can lead to a large polymer is now established.

What can be said about the mechanism of oligomer replication to produce a macromolecule? The most significant data available describe an optimal temperature distinctive for each oligomer in the series (Fig. 3). The fact that $(AT)_4$, for

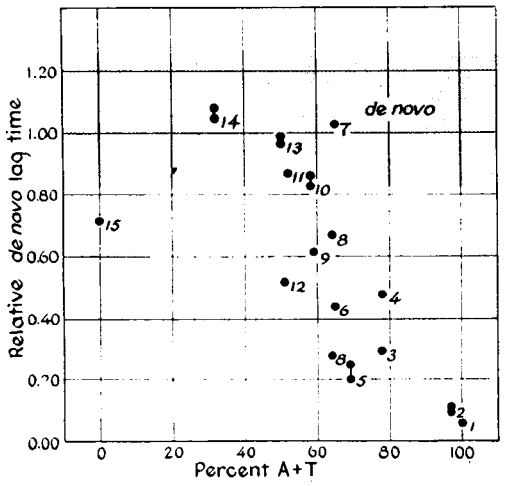

FIG. 4.—Influence of DNA on dAT synthesis. The reaction mixtures at 37° and as described in *Materials and Methods* contained polymer or DNA in the amount of 2 mμmoles (nucleotide residues) for (1) dAT and (2) crab testis, or 20 mμmoles (nucleotide residues) for (3) slime mold, (4) *T. patula*, (5) yeast cytochrome b_2, (6) *Paracentrotus lividus* (a sea urchin), (7) T2 phage, (8) whole yeast, (9) salmon sperm, (10) calf thymus, (11) T7 phage, (12) λ phage, (13) *E. coli*, (14) *Mycobacterium phlei*, and (15) dGdC. The lag time of *de novo* dAT synthesis (shaded area) was 360–545 min in an early series of experiments and 500–726 min in a later series; the median *de novo* value was set at 1.0 for each series. The double points for samples 2, 5, 8, 10, 13, and 14 represent separate experiments.

example, primes optimally at 10° while $(AT)_7$ is virtually inert at this temperature argues against the stepwise extension of an $(AT)_4$ primer chain through intermediate stages which include $(AT)_7$. Unfortunately, definitive evidence to establish this point is lacking and, as a consequence, several possible mechanisms can be entertained. A model which at this time appears plausible and offers a basis for discussion is described in Figure 5. The first step involves replication of the template with a new strand starting from the 3′-hydroxyl end of the template. The second step entails the melting of this newly formed helix and its annealing to expose a segment of template for further replication. This second step in which reunion of the separated strands is correct in base pairing but displaced by one AT notch from perfect realignment may be regarded as a slippage process. An important assumption at this point is that the DNA polymerase binds the template in a way that distinguishes it from the growing replica; it might also be assumed that such an enzyme complex of template and replica favors their union by hydrogen bonding. On the basis of this model we may consider that it is the slippage step of the reaction which is critically temperature-dependent. An $(AT)_4$ template after slippage would be linked to its replica by only 6 hydrogen bonds, a complex which would not be expected to be stable at 37° but might be far more stable and effective at 10°. By contrast, an $(AT)_7$ template linked by 12 hydrogen bonds to its slipped replica would be relatively more effective for replication at 37°. However, at 10° there is probably relatively little "breathing" in the $(AT)_7$ template-replica complex, and the system would therefore be almost frozen for further replication. This model, entailing successive steps of replication and slippage, would lead eventually to a continuous reiteration of the template and the development of a large dAT polymer. This scheme does not take into account the possibly important role played by helical hairpin structures which seem likely to develop in the growing replica (Fig. 5) and which may also be present in the larger oligomers, such as $(AT)_7$.

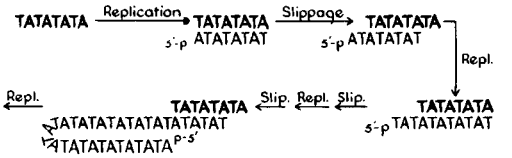

FIG. 5.—A model for reiterative replication of oligonucleotides. The $(AT)_4$ template is designated in *bold print*. Replication proceeds from left to right; the initial unit of the newly synthesized strand, designated by standard print, is marked as a 5′-phosphoryl end group (pA . . .).

A model for polymer synthesis involving replication and slippage was first proposed by Chamberlin and Berg[12] to account for the development of polyriboadenylate by RNA polymerase. Their suggestion that short sequences of deoxythymidylate in the DNA template were reiterated was supported by the demonstration by Falaschi, Adler, and Khorana[13] that a synthetic deoxypolythymidylate with only five residues could serve as a template for polyriboadenylate synthesis. It would be interesting to find out whether the reaction temperature would have the same kind of influence on the replication of deoxythymidylate oligonucleotides by RNA polymerase as that described in the present report for the AT series by DNA polymerase.

This model of reiterative replication by DNA polymerase has suggested several inquiries. One is the possibility that AT sequences in DNA would, in the absence of dGTP or dCTP, be reiterated sufficiently to prime the synthesis of a dAT poly-

mer. The results (Fig. 4) suggest that certain AT-rich DNA's do in fact prime dAT synthesis. While a clear interpretation of these results is not at hand, it seems reasonable that the inability to copy a chain for lack of a matching G or C would predispose the looping out of a preceding synthesized TAT sequence. Given the possibility of such TAT loops[14] and a reiteration of the ATA sequence in the template, the stage may be set for the initiation of an AT polymer. Conjectures of this kind lead to speculations that the origin of ATA or TAT sequences in natural DNA, so abundant in the crab testis DNA, for example, may be the consequence of such reiterative errors in replication. It follows that, if such reiterative errors have occurred with any frequency, they may be associated with distinctive mutational changes in amino acid sequences reflecting repeated ATA or TAT sequences.

Another line of inquiry generated by these studies concerns the use of temperature to explore the nature of DNA replication. Would the activation energy of the DNA polymerase reaction, given an exposed strand of DNA for replication, resemble that of other enzyme-catalyzed reactions with a $Q_{10°}$ of about 2? If, however, replication requires that a given stretch of double helical DNA first be separated into its component strands, would the reaction be critically dependent on temperature with the involvement of very large activation energies? Current experiments with DNA polymerase which distinguish the repair of a partially single-stranded helix from the synthesis of new strands provide an opportunity to test the influence of temperature on these two kinds of replication. Preliminary results[15] indicate that when the temperature is lowered from 37° to 20°, the repair process proceeds at a rate which is still a third to a half as great as at the higher temperature. By contrast, the new chain synthesis, which presumably requires the initial separation of the ends of the double helical template, is practically nil at 20°.

Summary.—(1) Oligonucleotides with sequences of 6–14 alternating deoxyadenylate and deoxythymidylate residues [$(AT)_3$ to $(AT)_7$] prime the synthesis by *E. coli* DNA polymerase of the high-molecular-weight deoxyadenylate-deoxythymidylate copolymer. At 37°, the relative priming capacity of this series of $(AT)_n$ oligomers was directly related to their size; the absence of a 5′-phosphoryl group from the end of certain oligomers had no apparent effect.

(2) Temperature has a profound influence on the replication of the oligomers; in the range 0–45° there is an optimal temperature distinctive for each oligomer in the series and a direct correlation between the size of the primer and the optimal temperature for its replication. For example, $(AT)_4$ is replicated most rapidly at 10°, $(AT)_5$ at 20°, and $(AT)_6$ at 37°.

(3) A model has been suggested in which replication of the oligomer template is followed by slippage of the resulting helix by an AT dinucleotide pair; such successive steps of replication and slippage many times would result in the continuous reiteration of the template and the synthesis of a large dAT copolymer. The possibility that AT sequences in DNA chains may also promote dAT synthesis is encouraged by the observed priming effect of several AT-rich DNA samples.

* This investigation was supported by research grants from the National Institutes of Health, USPHS.

† Foreign Scientist Research Fellow of the U.S. Public Health Service.

[1] Abbreviations used: A, adenine, deoxyadenosine or deoxyadenylate; DNA, deoxyribonucleic

acid; T, thymine, deoxythymidine, or deoxythymidylate; dATP, dCTP, dGTP, and dTTP, deoxynucleoside triphosphates of adenine, cytosine, guanine, and thymine, respectively. Designations for a polynucleotide chain are those described in *J. Biol. Chem.*

[2] Kornberg, A., in *The Chemical Basis of Heredity*, ed. W. D. McElroy and B. Glass (Baltimore: Johns Hopkins Press, 1957), p. 579.

[3] Weimann, G., H. Schaller, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3835 (1963).

[4] Schachman, H. K., J. Adler, C. M. Radding, I. R. Lehman, and A. Kornberg, *J. Biol. Chem.*, **235**, 3242 (1960).

[5] Radding, C. M., and A. Kornberg, *J. Biol. Chem.*, **237**, 2877 (1962).

[6] Swartz, M. N., T. A. Trautner, and A. Kornberg, *J. Biol. Chem.*, **237**, 1961 (1962).

[7] Richardson, C. C., C. L. Schildkraut, H. V. Aposhian, and A. Kornberg, *J. Biol. Chem.*, **239**, 222 (1964).

[8] Lehman, I. R., G. G. Roussos, and E. A. Pratt, *J. Biol. Chem.*, **237**, 819 (1962).

[9] Lehman, I. R., and C. C. Richardson, *J. Biol. Chem.*, **239**, 242 (1964).

[10] Baldwin, R. L., and E. M. Shooter, *J. Mol. Biol*, **7**, 511 (1963).

[11] The concentrations of oligomers used in these and later experiments permitted measurements within a convenient time span and were economical of the limited supplies of these compounds; however, it should be noted that these levels were below those required to saturate the enzyme.

[12] Chamberlin, M., and P. Berg, these PROCEEDINGS, **48**, 81 (1962).

[13] Falaschi, A., J. Adler, and H. G. Khorana, *J. Biol. Chem.*, **238**, 3080 (1963).

[14] Fresco, J. R., B. M. Alberts, and P. Doty, *Nature*, **188**, 98 (1960).

[15] Richardson, C. C., and A. Kornberg, unpublished results.