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On the Fidelity of DNA Replication

NUCLEOSIDE MONOPHOSPHATE GENERATION DURING POLYMERIZATION*

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During catalysis by homogeneous procaryotic DNA polymerases, nucleoside monophosphates are generated by a 3′ → 5′-exonucleolytic activity. Using *Escherichia coli* DNA polymerase I and poly[d(A-T)] as a template, the contribution of this activity to the fidelity of DNA synthesis has been evaluated by three different criteria. 1) The ratio between the rates of monophosphate generation and incorporation of the noncomplementary nucleotide with Mg^{2+} as an activating cation was 0.6 ± 0.6, which is insufficient to account for the high fidelity of polymerization. 2) Inhibition of polymerization by pyrophosphate fails to diminish fidelity, although some kinetic models suggest that optimal error correction via monophosphate release requires the polymerization reaction to be strongly driven by pyrophosphate release. 3) The addition of deoxynucleoside monophosphates in concentrations as great as 10 mM to the reaction mixture does not alter the fidelity of DNA synthesis. These observations argue against the kinetic proofreading mode to account for the fidelity of *E. coli* DNA polymerase I when copying poly[d(A-T)] in a Mg^{2+}-activated reaction. Furthermore, they suggest that the polymerase may enhance specificity at the base-selection step. However, the 3′ → 5′ exonuclease plays a larger role when the polymerase is activated with Mn^{2+} and may also be important in copying natural DNA where lower error rates are observed *in vitro*.

From an analysis of the structure of DNA, Watson and Crick (1) suggested that faithful duplication of base sequence was mediated primarily by hydrogen bonding between the nucleotides on the parental DNA and the nucleotides being polymerized on the daughter strand. However, the difference in free energy between correct and incorrect Watson-Crick base pairings is at most only 2 to 3 kcal/mol. This estimate is based on quantum mechanical calculations (2), measurements of nucleotide interactions (3), stability of polynucleotide helices containing varying numbers of noncomplementary base pairs (4, 5), and measurements of frequency of incorporation of nucleotide analogues by DNA polymerases (6). If Watson-Crick base pairing were the only free energy of discrimination available, the error frequency, f_0, would be 1 mispaired nucleotide in every 100 nucleotides incorporated (7–9). In a previous paper (10), we have confirmed and extended the initial observations of Trautner *et al.* (11) on the high fidelity of *Escherichia coli* DNA polymerase I. With poly[d(A-T)] as a template the error frequency *in vitro* can approach 1/80,000. Polymerases might increase fidelity by two different mechanisms: 1) by increasing the specificity of base selection prior to misinsertion ("error prevention") or 2) by a two-step mechanism in which noncomplementary nucleotides are excised after misinsertion ("proofreading").

Models to explain enhanced discrimination without excision have included template-induced changes in the conformation of the enzymes so that the substrate site will accommodate only the correct substrate (12–14) or the correct base pair (15, 16), base-specific nucleotide-binding subsites (17, 18), tightening of enzyme-template binding in the presence of the correct nucleotide (19), and preorientation of the enzyme-bound nucleotide into the required conformation prior to covalent incorporation (20). Support for models involving increased base selection rests heavily on the DNA polymerases from eucaryotic cells (21, 22), RNA tumor virus (23), and RNA polymerase (24) which lack the capacity to excise noncomplementary nucleotides and yet exhibit error rates much less than predicted by Watson-Crick base pairing alone.

Procaryotic DNA polymerases contain a 3′ → 5′ exonucleolytic activity (25, 26). Based on the preferential excision of noncomplementary nucleotides at the 3′-primer terminus and the need to excise these mismatched nucleotides prior to polymerization, Brutlag and Kornberg (27) postulated that the 3′ → 5′ exonuclease serves a proofreading function. This concept is supported by studies on mutants of bacteriophage T_4 with altered DNA polymerase (26, 28). DNA polymerases from some "mutator" strains have a reduced ratio of exonuclease to polymerase activity, whereas the polymerases from "antimutator" strains have an increased ratio of exonuclease to polymerase (28). However, monophosphate generation during the course of synthesis is a more relevant measure of proofreading than is exonuclease activity since the latter is measured in the absence of polymerization. Also, Hershfield (29) demonstrated that the infidelity by the purified mutator DNA polymerase from T_4 ts L88 resulted primarily from

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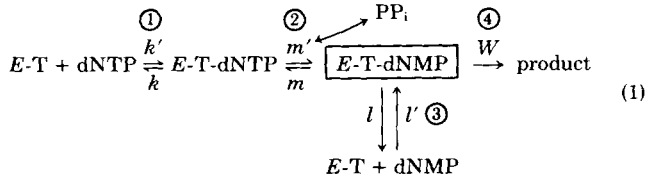
increased misinsertion and not from less frequent excision.

The concept of proofreading implies that there is a second discriminatory step by which the enzyme can correct any errors in base pairing. The first discriminatory step results from the energy difference between the formation of correct and incorrect base pairs. The second step results from the preferential hydrolysis of the incorrect base pairs. Hopfield (9) and Ninio (30) have formalized this concept into models for proofreading.

In Hopfield's model for kinetic proofreading, the nonspecific driving force of pyrophosphate release upon incorporation, coupled with the selective hydrolysis of noncomplementary nucleoside monophosphates leads to an error frequency approaching f_0^2, the square of the error fraction due to differences in base pairing.

The pathway proposed by Hopfield (9) is shown below:



(1)

Step 1 is a reversible reaction governed by rate constants k' and k, in which the deoxynucleoside triphosphates (dNTP) bind to the enzyme-template complex (E-T). This step discriminates against incorrect base pairing by the Watson-Crick error fraction, f_0. In Step 2 (governed by rate constants m' and m), pyrophosphate (PP_i) is released as the dNTP is attacked by the 3′-OH primer terminus. The step is nonspecific and essentially irreversible because of the large negative free energy of pyrophosphate release. In Step 3 (governed by rate constants l' and l), the boxed intermediate (hereafter referred to as "the enzyme-template-deoxynucleoside monophosphate intermediate" and abbreviated as E-T-M intermediate) may be hydrolyzed by the 3′ → 5′ exonuclease to yield free nucleoside monophosphates. Because of the large free energy of pyrophosphate release, the ETM intermediate may be postulated to be a high energy intermediate even though its formation via Step 2 is strongly favored. Thus Step 3 may be driven in the decomposition direction. Moreover, correct ETM intermediates decompose more slowly than incorrect ones by a factor of f_0. Finally, in Step 4 (governed by the rate constant W), the polymerase translocates so that the 3′-OH of the newly incorporated nucleotide occupies the primer terminus site on the enzyme. The rate constants with correct and incorrect substrates can be designated by subscripts C and D, respectively. Thus, l_C is the rate of decomposition of the correct ETM intermediate via Step 3. In the kinetic proofreading model (9), specificity is assumed to reside entirely in the "off" rates. Hence $k_C' = k'_D$ and $l'_C = l'_D$, but $k_C/k_D = f_0$ and $l_C/l_D = f_0$. To enable the base-specific steps to control the composition of the first activated complex, m' is assumed to be smaller than k_C or k_D. Moreover, W, which is assumed equal for both correct and incorrect nucleotides, is assumed small. Because Steps 1 and 2 are strongly driven in the forward direction and Step 3 is strongly driven in the decomposition direction, the two pathways become interdependent and the error rate approaches f_0^2.

In the proofreading scheme proposed by Ninio (30), the polymerase-template nucleotide intermediate is subject to a time delay in which the nucleoside monophosphate is subject to excision but may not be incorporated. This model is presented in the appendix (I) and compared to the model proposed by Hopfield (9).

In this paper, we examine the effects of inorganic pyrophosphate, nucleoside monophosphates and their analogues, and Mn^{2+} on fidelity, incorporation, and monophosphate generation by *E. coli* DNA polymerase I. We conclude that while monophosphate generation during polymerization may weakly promote additional fidelity, it is not responsible for the accuracy by which *E. coli* DNA polymerase I copies poly[d(A-T)]. To account for this accuracy we invoke enzymatically enhanced base specificity prior to misinsertion, although kinetic mechanisms for accuracy that do not manifest incorrect monophosphate generation cannot be eliminated. Alternatively, it is possible that previous studies (2–6) have underestimated Watson-Crick base pair energies. Finally, we note that monophosphate generation may play a major role in other situations, specifically in the presence of Mn^{2+} or in the copying of natural DNA. In the later situation, a higher fidelity is obtained, and this may be correlated with enhanced proofreading.[1]

MATERIALS AND METHODS

The materials used in this paper were obtained as described in the previous paper (10).

Preparation of Poly[d(A-T)] Labeled at the 3′-Terminus with Radioactive dTMP—Poly[d(A-T)] was labeled at the 3′-terminus by the limited addition of *E. coli* DNA polymerase I. The reaction was carried out in a 0.5-ml volume containing 50 mM Tris-HCl (pH 7.8), 2 mM $MgCl_2$, 10 μM [α-^{32}P]dTTP, (50×10^3 dpm/pmol), 10 μM dATP, 50 μg of poly[d(A-T)], and 2 nM Pol I.[2] Incubation was terminated after 2 min at 37 °C by the addition of 0.05 ml of unlabeled 50 mM dTTP and 0.05 ml of 50 mM EDTA. The product was isolated by phenol extraction and then dialyzed for 4 days against 4 liters of 50 mM Tris-HCl (pH 7.4) containing 1.0 M KCl and then against 1 liter of 50 mM Tris-HCl (pH 7.4) at 4 °C. On the basis of radioactivity incorporated, 0.3% of the poly[d(A-T)] template was copied.

Chromatography of Nucleoside Triphosphates—Deoxynucleoside triphosphates were purified immediately prior to use in assays for monophosphate generation by procedures selected for removing contaminating nucleoside monophosphates. Purification was either by chromatography on DEAE-cellulose (32) or by high pressure liquid chromatography. DEAE-cellulose was in the bicarbonate form, and elution was carried out with triethylammonium bicarbonate (pH 7.5–8.0); the concentration of the latter was 0.15–0.50 M for dCTP or dTTP and 0.3–0.5 M for dGTP and dATP. The triethylammonium bicarbonate was removed by evaporation under reduced pressure at 30–35 °C using increasing concentrations of methanol. Separation by high pressure liquid chromatography was carried out at 4 °C on a Spectra Physics model 8000, equipped with a double beam UV detector at a wavelength of 254 nm. The sample (250 μl) was loaded onto a Partisil 10 SAX column (Whatman, Clifton, NJ), and elution was carried out using a two-step linear gradient of 0.0035 M KPB (pH 7.6) (solution A) to 0.35 M KPB (pH 7.2), 0.6 M KCl (solution B) at a flow rate of 2 ml/min. During the first step (25 min), solution B was increased from 0 to 30%. In the second step, either 20 or 30 min, the increase was from 30 to 80%. Prior to nucleotide analysis, the column was washed by running through a gradient without added sample. After each run, the column was washed with 20 ml of solution B and then re-equilibrated with 30 ml of solution A. Each buffer solution (1 liter) was run through sequential columns (10 × 1 cm) of Chelex 100 and activated charcoal and then passed through a membrane filter (HA; Millipore, Bedford, MA). Fractions of 0.8 ml were collected. The peak fraction containing [^{3}H]dGTP was identified by measuring radioactivity and immediately used in fidelity assays or frozen in liquid nitrogen. The newly purified [^{3}H]dGTP was stable for up to 2 months in liquid nitrogen, *i.e.* no hydrolysis of dGTP to dGMP was detected during the period, the limit of detection being 10^{-6}. Attempts to remove salt and concentrate the [^{3}H]dGTP by gel filtration, lyophilization, chromatography on either DEAE-cellulose or activated charcoal, or evaporation at 20 °C under reduced pressure resulted in greater than 0.001% hydrolysis of dGTP to dGMP and thus was not acceptable.

[1] T. A. Kunkel, R. Shaaper, R. A. Beckman, and L. A. Loeb, manuscript in preparation.

[2] The abbreviations used are: Pol I, *Escherichia coli* polymerase I; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SSB, single strand binding protein.

Fidelity Assay—The standard fidelity reaction mixture (total volume 0.15 ml) contained 4 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 20 μM dATP, 20 μM [α-^{32}P]dTTP (4–20 dpm/pmol), 20 μM [^{3}H]dGTP (40,000 dpm/pmol), 6 μg of poly[d(A-T)], and 12 nM *E. coli* DNA polymerase I. Incubation was for 15 min, and incorporation of the radioactive nucleotide into an acid-insoluble precipitate was determined on 50-μl aliquots as previously described (33). The error rate is the ratio of noncomplementary to total complementary nucleotide incorporation.

Generation of Nucleoside Monophosphates—The amount of [^{3}H]dGMP generation was determined using 50-μl samples from each of the fidelity reactions. The reactions were terminated by adding 5 μl of 50 mM EDTA (pH 7.4) and 5 μl of 20 mM dGMP, 20 mM dGTP as markers to each sample. Thin layer chromatography was carried out in three directions using polyethyleneimine cellulose F (E. Merck, Darmstadt, Germany). Prior to use, the plates were washed with water by ascending chromatography. After drying, eleven 5-μl aliquots from each sample were applied at 4 °C repetitively in a single spot (~0.5 cm) located 2.5 cm from the corner of each plate. The spot was washed by agitation in 80% methanol at 4 °C. After drying, ascending chromatography in the first direction was carried out with 1.2 M LiCl, 0.005 M Tris-HCl (pH 7.8) for 5 h at 4 °C using an attached paper wick which allowed the solvent to travel ~28 cm. After locating dGTP and dGMP with a UV lamp, the plate was cut 1 cm below the dGMP spot. After soaking the portion of the plate containing dGMP in 80% methanol for 15 min, the plate was allowed to dry and subjected to ascending chromatography in the second direction perpendicular to the first with 1.4 M $LiCl_2$, 0.025 M Tris-HCl (pH 7.8) for 3.5 h at 20 °C. The plate was cut 3 cm below dGMP, washed in methanol, and then chromatographed with 70% methanol for 1 h in the direction opposite to the second. The latter procedure separated any radioactivity that migrated with the solvent front. The spot migrating with dGTP in the first direction was cut out and eluted by soaking the plate fragments in 5 ml of 2.0 M LiCl and counting a 50-μl aliquot in Aquasol. The spot migrating with dGMP was cut into 1-cm squares and placed into a counting vial to which was added 1.5 ml of 1.0 M LiCl. After agitation for 5 min, 12 ml of Aquasol were added. In order to calibrate the system, three samples of 50 μl of 1 mM [^{3}H]dGMP containing 5×10^3 cpm was chromatographed. Recovery of radioactivity in the dGMP spot was 91, 94, and 98% of that applied.

RESULTS

E. coli polymerase I (Pol I) is highly accurate in copying poly[d(A-T)]; the frequency of [^{3}H]dGTP misincorporation with Mg^{2+} with different batches of Pol I and labeled substrates varied from 1/15,000 to 1/80,000. However, in replicate determinations with the same template, substrate, and enzyme preparation, the error rate varied by less than 5%. The rate of incorporation of the complementary and noncomplementary nucleotide is linear for at least 30 min of incubation using concentrations of Pol I between 0.1 and 10 nM.

Ratio of Monophosphate Generation to Incorporation—For an analysis of editing during polymerization, proofreading, the ratio of exonuclease to polymerase may not be the most pertinent. Exonuclease activity is measured in the absence of polymerization. Exonucleolytic hydrolysis occurs on preformed phosphodiester bonds and thus may not reflect monophosphate generation from an *E*TM intermediate. Measurement of monophosphate generation can be directly equated with excision during the course of polymerization. Table I shows the rates of stable incorporation and monophosphate generation for both complementary and noncomplementary nucleotides using poly[d(A-T)] as a template and Mg^{2+} as a metal activator. In these experiments, all deoxynucleoside triphosphates were present in equal concentration, 20 μM, which approximates the K_m of the complementary nucleotide. With [α-^{32}P]dATP as the labeled substrate, the rate of polymerization in the fidelity assay approximates 60 mol of total nucleotides incorporated/mol of enzyme protein/min. This rate is less than that reported under conditions in which all components of the reaction are present in saturating amounts, 200–1000 nucleotides per min (15). The molar ratio of monophosphate generation to incorporation for the complementary nucleotide is constant for up to 45 min of incubation and varies in different experiments from 0.11 to 0.19. This ratio is not greatly different from the value of 0.22 obtained by Radman *et al.* (34).

Measurements of noncomplementary nucleoside monophosphate generation are technically difficult due to the large excess of the noncomplementary nucleoside triphosphates in the reaction mixture and the spontaneous breakdown of nucleoside triphosphates to monophosphates. In a typical fidelity assay, the incorporation of the noncomplementary nucleotide (error rate, 1/20,000 to 1/80,000) represents a minute per cent of the labeled substrate in the reaction mixture. With [α-^{32}P]dGTP as the noncomplementary substrate and Mg^{2+} as the metal activator, the molar ratio of monophosphate generation to stable incorporation was less than 0.5. However, the error rate with [α-^{32}P]dGTP has been observed to be high (1/6,000) in multiple experiments. With [^{3}H]dGTP, purified by DEAE-cellulose, no monophosphate generation was detected above background; the ratio of generation to incorporation could only be estimated to be less than 2.0. Thus, less than two dGMP's are generated for each one incorporated (*vide infra*).

Studies with Mn^{2+}—Manganese, a well known mutagen (37), has been shown to increase misincorporation during

TABLE I

Rates of monophosphate generation and incorporation during polymerization

Incorporation and monophosphate generation were determined as described under "Materials and Methods" after incubation for 0, 10, 20, 30, 45, and 60 min. Each nucleotide was present at 20 μM, and all assays were carried out in triplicate. The specific activities in dpm/pmol are as follows: [α-^{32}P]dATP, 176, [α-^{32}P]dGTP, 44,000, and [^{3}H]dGTP, 25,000. [^{3}H]dGTP was purified by DEAE-cellulose (25) immediately before being used in the fidelity reaction. Contamination of [^{3}H]dGTP with [^{3}H]dGMP was determined by thin layer chromatography and found to be less than 0.01%. The values given are for incubation of 10 min; the ratio was obtained from the slope of the rates of incorporation and monophosphate generation. Where indicated, 0.1 mM $MnCl_2$ was used in place of $MgCl_2$ as a metal activator. A stock solution of 0.1 M $MnCl_2$ was made in 0.01 N HCl and stored in plastic containers at 0 °C. The concentration of free Mn^{2+} in the stock solution was determined from the amplitude of the electron paramagnetic resonance spectrum (36). Monophosphate generation was measured by spotting 15 μl of each fidelity reaction onto polyethyleneimine cellulose thin layer plates and then separating the nucleotides by one-dimensional chromatography in 1.8 M LiCl as described by Hershfield and Nossal (35).

Deoxynucleotide added		Metal activator	Stable incorporation	Monophosphate generation	Ratio of monophosphate generation to incorporation
Labeled	Unlabeled		*mol labeled nucleotide/mol polymerase/min*	*mol labeled monophosphate/mol polymerase/min*	
[α-^{32}P]dATP	dTTP, dGTP	Mg^{2+}	31.1	5.33	0.17
[α-^{32}P]dGTP	dTTP, dATP	Mg^{2+}	0.011	<0.005	<0.5
[^{3}H]dGTP	dTTP, dATP	Mg^{2+}	0.003	<0.006	<2.0
[α-^{32}P]dATP	dTTP, dGTP	Mn^{2+}	5.55	4.26	0.8
[α-^{32}P]dGTP	dTTP, dATP	Mn^{2+}	0.012	0.47	39.1

catalysis by DNA polymerases from various sources (22). We have previously shown that substitution of Mg^{2+} by Mn^{2+} decreases fidelity of Pol I without reducing the exonucleolytic activity as measured with templates labeled at the 3'-terminus (38). Substitution of Mn^{2+} for Mg^{2+} increases the rate of both correct and incorrect monophosphate generation. With respect to the noncomplementary nucleotide, monophosphate generation was 39 times greater than that of stable incorporation (Table I). In the context of this high error rate, 1/926, monophosphate generation is sufficient to account for the enhancement of fidelity above our estimate for Watson-Crick base pairs. Thus, under mutagenic conditions, kinetic proofreading may play a major role in achieving accuracy.

Purification of [^{3}H]dGTP—In order to more precisely measure dGMP generation, it was necessary to extensively purify [^{3}H]dGTP. The separation of dGMP from dGTP by high pressure liquid chromatography is shown in Fig. 1. In a typical experiment, 15 mCi of [^{3}H]dGTP was dissolved in 250 μl of 20 mM Tris-HCl (pH 7.8) and loaded onto a Partisil 10 SAX column. The peak fraction containing dGTP was used in experiments that measure monophosphate generation. Rechromatography of the purified dGTP by high pressure liquid chromatography and by three-directional thin layer chromatography indicated that dGMP contamination of the purified dGTP to be 1.5×10^{-5} and 0.4×10^{-5}, respectively. The difference may indicate some hydrolysis of dGTP during repeated high pressure liquid chromatography.

Analysis of the Poly[d(A-T)] Template—The poly[d(A-T)] used in these experiments was synthesized with Pol I by the *de novo* polymerization of dATP and dTTP in the presence of trace amounts of [^{3}H]dGTP (10). By measuring the amount of [^{3}H]dGTP incorporated and the amount of contaminating unlabeled dGTP in the reaction mixture, it was concluded that poly[d(A-T)] was contaminated with only 1 in 2×10^6 mol of dGMP/mol of total nucleotide. Unfortunately, a similar analysis cannot be carried out with dCTP due to deamination (39). However, by three criteria, we estimate that dCMP contamination of poly[d(A-T)] is equal to or less than 1 in 200,000. 1) In copying poly[d(A-T)] with Pol I with total nucleotide incorporation of 320 pmol, dGTP incorporation was 0.005/pmol, while with T_4 DNA polymerase, dGTP incorporation was 0.0016 pmol. This yields an error rate for T_4 DNA polymerase of 1/210,000, indicating that the frequency of dCMP residues in the template is less than 1 in 200,000. 2) The addition of *E. coli* SSB protein to reactions catalyzed by Pol I decreased the error rate from 1/32,000 to 1/133,000 (40). Since *E. coli* SSB protein also increases the fidelity of copying natural DNA templates (40), it is unlikely that the effect observed with poly[d(A-T)] is due to nonspecific blockage by SSB at dCMP residues contaminating the template. 3) Incorporation of dGTP can be reduced by the addition of unlabeled dATP in an approximately linear manner, so as to achieve error rates as low as 1 in 143,000 (Table II).

Studies with Purified Components—In order to accurately quantitate dGMP generation, we utilized [^{3}H]dGTP purified by high pressure liquid chromatography to contain less than 10^{-5} parts of dGMP. Furthermore, enhanced sensitivity was achieved by reducing the concentration of dATP 4-fold and thus increasing the frequency of misincorporation (Table III). At equal nucleotide concentrations (20 μM), the error rate was 1/64,000; decreasing dATP to 5 μM increased the error rate to 1/17,200. With Mg^{2+}, the results on four separate analyses with two batches of purified [^{3}H]dGTP indicate that 0.6 ± 0.6 mol of dGMP was generated for each dGMP incorporated. Since all experimental values were less than two times background, we can assume only that the true amount of incorrect monophosphate generated is less than or equal to 1.2 mol of dGMP/mol incorporated. In this experiment, the error rate was 1/68,800. Since at most 1.2 mol of dGMP is hydrolyzed for every 1 mol incorporated (*i.e.* 1.2 hydrolyzed for every 2.2 mistakes), monophosphate generation can correct only about 55% of the mistakes. In order to achieve an accuracy of 1/68,800, the misinsertion frequency could be reduced by proofreading to 1/30,500, a value lower than can be achieved by Watson-Crick base pairing. Thus, while the generating of incorrect monophosphates is compatible with a role for proofreading by Pol I in accuracy, it also indicates that proofreading alone cannot account for the high fidelity observed. The fact that the dGMP generation could be detected is demonstrated by the enhancement of dGMP generation with Mn^{2+}. T_4 DNA polymerase is known to exhibit high level exonucleolytic activity (19). With this enzyme, the ratio of incorrect monophosphate generation to incorporation is 110 to 1, and this is adequate for proofreading.

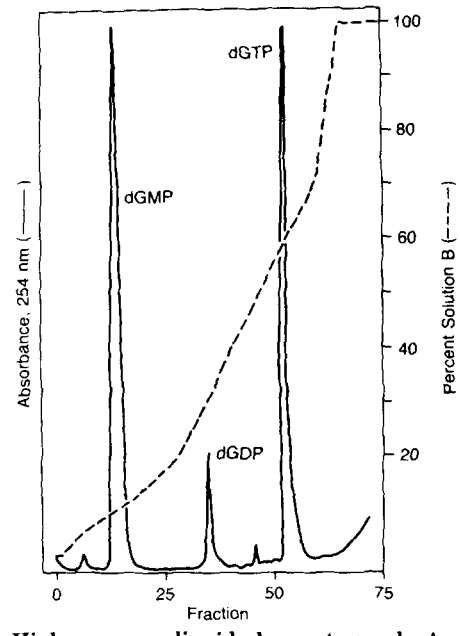


FIG. 1. **High pressure liquid chromatograph.** A sample (250 μl) of 100 nmol of [^{3}H]dGTP and dGMP in 20 mM Tris-HCl (pH 7.5) was separated on a Partisil 10 SAX column as described under "Materials and Methods." The tube containing the highest concentration of [^{3}H]dGTP (fx 53) was used in assays measuring monophosphate generation (Table II). Repeat chromatography yielded an almost identical pattern. After all separation of dGTP was completed, the same column was used for determining the position of dTTP and dATP markers, which were eluted as single sharp peaks corresponding to fractions 42 and 48, respectively.

TABLE II

Effect of dATP on dGMP incorporation into poly[d(A-T)]

The reaction mixture (total volume 0.15 ml) contained 20 mM Tris-HCl (pH 7.8), 1.5 mM $MgCl_2$, 30 μM [α-^{32}P]dTTP (80 dpm/pmol), 30 μM [^{3}H]dGTP (40×10^3 dpm/pmol), 3 μg of poly[d(A-T)], and 9.2 nM Pol I, and the indicated amounts of dATP. Incubation was for 15 min at 37 °C, and [^{3}H]dGTP incorporation was corrected for cross-over from [α-^{32}P]dTTP. Incorporation of dGMP with added Pol I was 0.0015 pmol.

dATP	dGMP incorporated	dTMP incorporated	Error rate
μM	*pmol*		
30	0.042	364	1/17,000
50	0.023	344	1/30,000
90	0.018	391	1/43,000
150	0.010	340	1/68,000
200	0.006	275	1/92,000
300	0.006	427	1/143,000

TABLE III

Quantitation of dGMP generation

Fidelity reactions were carried out as described under "Materials and Methods." $MgCl_2$ and $MnCl_2$ were present at 1.5 and 0.7 mM, respectively. The concentration of dATP was 5 μM, and [^{3}H]dGTP was 20 μM or 13 μM, where indicated (*). In four separate experiments, the amount of dGMP generated minus zero-time controls with 16 nM of Pol I was 0.0, 0.008, 0.010, and 0 pmol. In the same experiments, the zero-time controls were 0.005, 0.008, 0.011, and 0.016 pmol, respectively.

DNA polymerase	Metal ion	Total nucleotide incorporated	dGMP incorporated	Extrapolated error rate	dGMP generated
			pmol		*pmol*
16 nM Pol I	Mg^{2+} (10 min)	132.5	0.0077	1/68,800	0.0045 ± (0.005)
32 nM Pol I	Mg^{2+} (5 min)	64.8	0.0066	1/39,300	0.009
25 nM Pol I	Mn^{2+} (20 min)	73.6	0.1200	1/2,400	5.1
32 nM Pol I*	Mg^{2+} (8 min)	184	0.0056	1/85,000	<0.003
T_4*	Mg^{2+} (15 min)	148	0.0016	1/240,000	0.18

Studies with Pyrophosphate—In order to study pyrophosphate inhibition without complexation of Mg^{2+}, the reaction was carried out in phosphate buffer. Deutscher and Kornberg (41) initially observed that maximum rates of polymerization with poly[d(A-T)] in phosphate buffer are obtained at a high Mg^{2+} concentration, about 7 mM. We have confirmed their observation and also noted that changing the Mg^{2+} concentration from 1 to 10 mM in the fidelity assays resulted in less than a 10% change in the rate of polymerization. This is in contrast to assays in Tris-HCl buffer in which a sharp maximal incorporation is observed at 1.0 mM Mg^{2+}. The effect of added pyrophosphate on polymerization and fidelity is shown in Table IV. At a concentration of 1.6 mM pyrophosphate, polymerization was reduced by 90% without decreasing fidelity. By the addition of a 10-fold greater amount of Pol I, the extent of synthesis was the same as in the absence of pyrophosphate, and the fidelity was not diminished. Inhibition by pyrophosphate appears to reverse polymerization; Deutscher and Kornberg (41) demonstrated that labeled pyrophosphate is incorporated into deoxynucleoside triphosphates, and the inhibition of polymerization can be accounted for by the extent of pyrophosphate exchange.

The error frequency f_0^2 derived from kinetic proofreading depends on the interdependence of the two reaction pathways in the Hopfield scheme, 1-2 and 3, each with an error frequency of f_0. Pathway 1-2 is driven toward formation of the *E*TM intermediate and 3 toward its hydrolysis. When the addition of excess pyrophosphate makes pathway 1-2 effectively reversible, this interdependence collapses, and one approaches the limiting case of two independent, reversible reactions, each with error fractions, f_0. Thus, inhibition of synthesis by the addition of pyrophosphate should progressively increase the error frequency from f_0^2 to f_0. We find no change in error frequency even though polymerization is decreased 90% in 1.6 mM pyrophosphate (Table IV).

These results argue against even a 2-fold correction using proofreading by a Hopfield mechanism (7) in the absence of "peel-back", *i.e.* the successive removal of several previously incorporated nucleotides by hydrolysis or pyrophosphorolysis. The irreversibility of Step 4 would preclude peel-back. Moreover, in the presence of peel-back we do not predict a decrease in fidelity by pyrophosphate. A decrease in error correction immediately after the insertion of the n^{th} nucleotide is compensated for due to enhanced peel-back by pyrophosphorolysis so that the polymerase is more likely to backtrack several times increasing the probability of error correction.[3] Thus, limited proofreading is compatible with our results if we modify Hopfield's model (9) to allow peel-back.

A quantitative assessment of the impact of pyrophosphate on fidelity in the absence of peel-back is presented in Appendix III. An analysis of the effect of increased peel-back on fidelity has been developed by Bernardi *et al.* (42).

[3] R. A. Beckman, unpublished calculations.

TABLE IV

Effect of pyrophosphate on the fidelity of E. coli DNA polymerase I

Fidelity assays were carried out as described under "Materials and Methods" except that 20 mM potassium phosphate (pH 7.4) was used as a buffer and 10 mM Mg^{2+} was the metal activator. PP_i was added as sodium pyrophosphate at the indicated final concentration. Incubation was for 14 min.

Addition	Incorporation		Error rate
	[α-^{32}P]dTMP	[^{3}H]dGMP	
m M	*pmol*		
None	119	0.004	1/59,500
PP_i (0.1)	98	0.004	1/49,000
PP_i (0.4)	138	0.005	1/55,000
PP_i (0.8)	35	0.001	1/70,000
PP_i (1.6)	14	0.0005	1/56,000
PP_i (1.6)[a]	120	0.004	1/60,000

[a] In this reaction the concentration of Pol I was 40 nM, 10-fold greater than in the standard assay.

Effects of Monophosphates and Their Analogues on Fidelity—Byrnes *et al.* (43) reported that various nucleoside monophosphates inhibit the exonucleolytic activity of Pol I and possibly monophosphate generation. It is thus of interest to determine the effect of nucleoside monophosphates on fidelity. An analysis of the effect of monophosphates on 3′ → 5′-exonucleolytic activity of Pol I upon poly[d(A-T)] with [^{3}H]dTMP at the 3′-OH termini is given in Table V. In experiment 1, incubation was for 15 min at 37 °C, the buffer was 50 mM potassium phosphate, and 10 mM Mg^{2+} was the metal activator. Only with 60 mM 5′-AMP was significant inhibition observed, 22%; less than 10% inhibition was observed with 20 mM dAMP, dGMP, dCMP, or with 30 mM 5′-AMP. Also, the rate of hydrolysis of poly[d(A-T)], terminated at the 3′-end with [α-^{32}P]dTMP, was not diminished by 10 mM AMP, 20 mM dGMP, and 20 mM dTMP (results not shown). Byrnes *et al.* (43) reported the inhibition of the 3′ → 5′ exonuclease of Pol I by nucleoside monophosphates in the presence of $MnCl_2$. The extent of hydrolysis was reduced with a variety of monophosphates (0.33 mM) to 10–40% of that obtained in the absence of added monophosphates. Even though the conditions in our experiments and those of Byrnes *et al.* (43) are not exactly comparable, we find little inhibition of hydrolysis by the 3′ → 5′-exonuclease by nucleoside monophosphates with Mg^{2+} in phosphate buffer (Table V, experiment 1) or with Mn^{2+} in Hepes buffer (Table V, experiment 2). Only with 60 mM 5′-AMP and Mg^{2+} or with 10 mM 5′-AMP and Mn^{2+} was significant inhibition obtained. More pertinently, no decrease in the fidelity of synthesis was observed with dGMP, 6-mercaptopurine ribonucleotide, or even 5′-AMP (Table VI). Thus, 5′-AMP reduces the 3′ → 5′-exonuclease activity yet fails to affect fidelity. It should be noted that the Hopfield model (9) predicts only a small decrease in error rate upon inhibition by monophosphate while the decrease would be greater on the basis of the scheme of Ninio

TABLE V

Effect of nucleoside monophosphates on the 3′ → 5′ exonuclease activity of E. coli DNA polymerase I

Each assay contained in a volume of 50 μl the following: 0.5 μg of poly[d(A-T)] labeled at the 3′-terminus with [^{3}H]dTMP, 0.08 M KCl, and 0.2 mM Pol I. In Experiment 1, the buffer was 50 mM potassium phosphate (pH 7.2), and the metal activator was 10 mM Mg^{2+}. In Experiment 2, the buffer was 50 mM Hepes (pH 7.0), and the metal activator was 0.5 mM Mn^{2+}. All reactions were carried out for 15 min at 37 °C, and the amount of [^{3}H] rendered acid soluble was determined as previously described (38).

Nucleoside monophosphate added	[^{3}H] rendered acid soluble	Activity
	cpm	%
Experiment 1		
None	1,350	100
5′-AMP (30 mM)	1,230	91
5′-AMP (60 mM)	1,054	78
dAMP (10 mM)	1,550	114
dAMP (20 mM)	1,335	99
dGMP (10 mM)	1,425	105
dGMP (20 mM)	1,332	99
dGMP (10 mM)	1,250	93
Experiment 2		
None	1,762	100
5′-AMP (1.0 mM)	1,580	90
5′-AMP (1.0 mM)	1,818	103
5′-AMP (10 mM)	1,193	60
dAMP (1 mM)	1,890	107
dAMP (10 mM)	1,655	93
6-MRPP[a] (1 mM)	1,921	109
6-MRPP (10 mM)	1,758	99

[a] 6-MRPP, 6-mercaptopurine ribonucleotide.

TABLE VI

Effect of different nucleoside monophosphates on the fidelity of DNA synthesis by E. coli DNA polymerase I using poly[d(A-T)] as template

Reaction conditions are the same as described under "Materials and Methods" using 0.1 M Tris-HCl (pH 7.4) as buffer. In Experiment III the concentration of Pol I was increased to 8 nM. Incorporation was insufficient for measurements of fidelity in Hepes buffer.

Addition	Incorporation		Error rate
	[α-^{32}P]dATP	[^{3}H]dGMP	
	pmol		
Experiment I			
None (10 mM Mg^{2+})	361	0.034	1/21,000
dGMP (10 mM)	423	0.032	1/26,000
dGMP (20 mM)	338	0.028	1/24,000
5′-AMP (2.5 mM)	310	0.023	1/27,000
5′-AMP (5 mM)	303	0.020	1/30,000
5′-AMP (10 mM)	281	0.013	1/42,000
6-MRPP[a] (1.6 mM)	330	0.022	1/30,000
6-MRPP[a] (4.0 mM)	172	0.011	1/31,000
Experiment II			
None (0.1 mM Mn^{2+})	108	0.047	1/4,600
dGMP (10 mM)	179	0.081	1/4,400
dAMP (10 mM)	173	0.056	1/6,500
5′-AMP (10 mM)	98	0.051	1/3,800
6-MRPP (10 mM)	100	0.036	1/5,600
Experiment III			
None (0.8 mM Mn^{2+})	50	0.106	1/944
dGMP (10 mM)	39	0.091	1/858
dAMP (10 mM)	53	0.098	1/1,100
5′-AMP 10 mM)	31	0.058	1/1,100
6-MRPP (10 mM)	27	0.060	1/900

[a] 6-MRPP, 6-mercaptopurine ribonucleotide.

(30). A quantitative assessment of this effect is given in the "Appendix."

DISCUSSION

We have tested a number of predictions of proofreading mechanisms as a source of fidelity using *E. coli* DNA polymerase I and poly[d(A-T)] as a template. We have previously presented evidence that this template is contaminated with only 1 in 2×10^6 mol of dGMP/mol of total nucleotide and that the accuracy of DNA synthesis by Pol I approaches 1/80,000 (10). We have further shown in this paper that dCMP contamination is less than 1 in 200,000 based on a number of criteria including the accuracy observed with T_4 DNA polymerase, 1/200,000. We find that the ratio of incorrect deoxynucleoside monophosphate generation to incorporation during synthesis with *E. coli* DNA polymerase I is insufficient to account for the accuracy obtained by proofreading alone. In addition, nucleoside monophosphates and their analogues only marginally inhibited the 3′ → 5′-exonuclease activity of Pol I and did not diminish the fidelity of DNA synthesis. Also, inhibition of DNA synthesis was achieved by inorganic pyrophosphate, but this did not increase errors, suggesting that proofreading cannot occur by a strict Hopfield mechanism (9) unless the scheme is modified to allow peel-back.

Byrnes *et al.* (43) reported that a variety of ribo- and deoxyribonucleoside monophosphates inhibited the exonucleolytic activity of *E. coli* DNA polymerase I. Inhibition of the exonuclease was accompanied by an increase in the rate of synthesis. A direct comparison between the results of Byrnes *et al.* (43) and ours is not possible. Their assays were carried out in the presence of 0.2 mM $MnCl_2$ and at low substrate concentrations. However, we observed no inhibition of exonuclease activity even in the presence of $MnCl_2$ under conditions in which the amount of added monophosphate was 500 times greater than that of the triphosphate substrates. Byrnes *et al.* (43) also report that 5′-AMP promotes misincorporation of dGMP into poly[d(A-T)] with Pol I in the presence of Mn^{2+}. With Mg^{2+} or Mn^{2+} we find no increase in misincorporation by this monophosphate. The results of Byrnes *et al.* (43) were obtained under conditions which artificially highlight the role of the exonuclease. Firstly, they measured noncomplementary nucleotide incorporation in separate reactions in the absence of the complementary nucleotide. Thus, their measurements of misincorporation concern the accuracy of terminal addition and may not be related to fidelity. Moreover, their low substrate concentration further reduces incorporation. Finally, their use of Mn^{2+} as a metal activator increases the rate of noncomplementary monophosphate generation (see Tables I and III). Thus, under the conditions of Byrnes *et al.* (43), kinetic competition is drastically altered to favor monophosphate generation, and the exonuclease may play a greater role in fidelity. Since their error rates are at least 100-fold higher than ours, we argue that the additional fidelity obtained under more physiologically relevant conditions is not explained by monophosphate generation.

If proofreading via a 3′ → 5′ exonuclease is not the only mechanism central to achieve fidelity, then *E. coli* DNA polymerase I must exhibit high specificity prior to insertion by utilizing the various energies involved in binding to the template-primer and deoxynucleoside triphosphates. This may occur by 1) preorienting the substrate toward the template in a specific conformation (20), such that the net discrimination energy associated with template-substrate interactions is increased; 2) a template-mediated conformational change, in which the enzyme preferentially binds the correct substrate (44); or 3) a tightening of enzyme-template binding in the presence of correct substrate (19). Any of these three methods would lead to greater insertion specificity due to increased discrimination energy between correct and incorrect *E*TM intermediates.

In addition to proofreading the 3′ → 5′ exonuclease of Pol I may have a role in postreplicative correction of errors or repair of damage. Perhaps completed duplex DNA is patrolled

by several nickases which recognize minor irregularities in the helix such as those caused by mispaired bases. Recently, evidence has accumulated for the repair of mistakes in base pairing by endonucleases present in procaryotes (31, 45). These endonucleases could hydrolyze the mispaired regions on the 3'-side. The 3' → 5' exonuclease of Pol I could then remove the misincorporated bases, and the same enzyme could polymerize and fill in the gap just created. This function for the 3' → 5' exonuclease is consistent with Pol I's function as a repair enzyme. Also, such a function is compatible with the correlation between the level of exonuclease activity and DNA polymerization in mutant T_4 DNA polymerases (28).

In this paper, we have considered how DNA polymerases decrease the error rate of 1/100 predicted from Watson-Crick base pairing to 10^{-5}, achieved during copying of poly[d(A-T)] *in vitro*. Our results indicate that the 3' → 5' exonucleolytic activity associated with *E. coli* DNA polymerase I is not kinetically competent to account for the fidelity of copying poly[d(A-T)]. Since the copying of natural DNA by Pol I may be more accurate, the 3' → 5' exonucleolytic activity may play a more important role. It is conceivable that proofreading by the 3' → 5' exonuclease is ineffective with homopolymers and polynucleotide templates that have an alternating structure which permit slippage during the course of polymerization (46). Repetitive nucleotide sequences are present in the genome and could be hypermutable on the basis of decreased proofreading. Long time delays associated with ATP-dependent unwinding of duplex DNA might allow more time for exonuclease to function during synthesis. Such a mechanism is suggested from studies on the CB120 anti-mutator polymerase from T_4, which is defective in strand displacement (47). The analysis in this paper provides an experimental approach for quantitating the contribution of proofreading to fidelity. Changes in accuracy using natural DNA templates by the addition of nucleoside monophosphates and pyrophosphate may permit one to assess the validity of various proofreading mechanisms.

With respect to isolated polymerases, the contribution of proofreading to fidelity varies considerably. Eucaryotic DNA polymerases are devoid of any exonucleolytic activity, fail to generate nucleoside monophosphates, and yet are able to copy polynucleotides with high fidelity (21, 22). Initial measurements with φX174 DNA indicate that substitution of dC for a dT at position 587 with DNA polymerases α and β occurs at a frequency of 1 in 30,000 and 1 in 3,000, respectively.[4] Enhanced binding specificity rather than proofreading presumably accounts for the accuracy of these polymerases. With φX174 DNA, the error frequency of Pol I at position 587 has been estimated to be 1 in 6×10^5 (48), and T_4 appears even more accurate (2). This accuracy might in part be the result of proofreading. With T_4 DNA polymerase the ratio of nucleoside monophosphate generation to incorporation is high enough to account for its fidelity using kinetic proofreading (19) even with poly[d(A-T)] as a template. Nonetheless, even for the T_4 polymerase there is evidence of enzyme-enhanced binding specificity. The temperature-sensitive mutator polymerase, *ts*L88, has been shown to have the same ratio of nucleotide monophosphate generation to incorporation as the wild type polymerase (19). Thus, the relative significance of enhanced base selection and kinetic proofreading in determining the fidelity of DNA synthesis may vary with different DNA polymerases and with different templates. The analysis in the appendix to this paper provides a framework for quantitating the contributions of kinetic proofreading to fidelity.

[4] T. A. Kunkel and L. A. Loeb, unpublished results.

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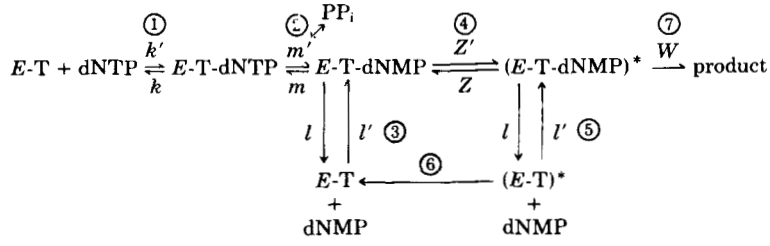
APPENDIX

I. Alternate Model for Kinetic Proofreading

Ninio (30) has independently formulated a kinetic proofreading model which differs slightly from that proposed by Hopfield (9). In the model proposed by Ninio (30), the *E*-T-M must undergo an unspecified change prior to incorporation of the nucleoside monophosphate. This could involve a change in the conformation of the enzyme or the binding of a second substrate. This unspecified event causes a time delay, during which time the bound nucleoside monophosphate is vulnerable to excision but cannot be incorporated.

We may formulate Ninio's model (30) in an expression analogous to that presented by Hopfield as follows,



(2)

Reactions 1, 2, and 3 are the same as in the Hopfield model. In reaction 4, governed by the rate constants Z' and Z, the unspecified change occurs in the *E*-T-dNMP intermediate (*E*TM intermediate). The new (*E*-T-dNMP)* intermediate may decompose by reaction 5, which is analogous to reaction 3, and is governed by the same rate constants. However, reaction 5 yields (*E*-T)*, the modified form of the enzyme-template complex. Without the conformational effect resulting from the presence of dNMP, (*E*-T)* is unstable and is converted irreversibly to *E*-T via reaction 6. Reaction 7, governed by the rate constant W, is the incorporation of dNMP from (*E*-T-dNMP)* into product.

In this model the excision step may discriminate by a factor of f_0^2 even when $l_C/l_D = f_0$. To illustrate this, let us define $\tau_C = 1/l_C$ and $\tau_D = 1/l_D$ as the mean survival times for the correct and incorrect (*E*T-dNMP)* intermediates, respectively, before hydrolysis. The number, n, of molecules of dNMP incorporated in time τ is equal to the integral overtime of the rate constant W times the instantaneous concentration of the (*E*-T-dNMP)*

$$n = \int_0^\tau W[(E\text{-T-dNMP})^*(t)]dt \tag{3}$$

Thus,

$$n_C = \int_0^{\tau_C} W[(E\text{-T-dNMP}_C)^*(t)]dt \tag{4}$$

and

$$n_D = \int_0^{\tau_D} W[(E\text{-T-dNMP}_D)^*(t)]dt \tag{5}$$

If τ_C and τ_D are short enough so that only a small fraction of the (*E*-T-dNMP) will be modified within these times, they will be within reaction 4's linear time range. It should be noted that our data contradict this fundamental assumption. If τ_C and τ_D were really much less than the time required to modify the *E*TM intermediate,

then monophosphate generation would predominate over incorporation, contrary to the data in Tables I and III.

$$[(E\text{-T-dNMP}_C)^*(t)] = Z'[(E\text{-T-dNMP}_C(0)]t \tag{6}$$

and

$$n_C = \int_0^{\tau_C} WZ'[E\text{-T-dNMP}_C(0)]t dt = \frac{WZ'[E\text{-T-dNMP}_C(0)]\tau_C^2}{2} \tag{7}$$

Similarly,

$$n_D = \frac{WZ'[E\text{-T-dNMP}_D(0)]\tau_D^2}{2} \tag{8}$$

The discriminatory factor offered by the excision step is

$$\frac{\dfrac{n_D}{[E\text{-T-dNMP}_D(0)]}}{\dfrac{n_C}{[E\text{-T-dNMP}_C(0)]}} = \frac{\tau_D^2}{\tau_C^2} = \frac{l_C^2}{l_D^2} = f_0^2 \tag{9}$$

In this model, it is not necessary that reactions 3 and 5 be driven in the decomposition direction, nor that the sequence 1–2 be driven in the forward direction by pyrophosphate release. The fidelity arises from the time delay step, reaction 4. It is necessary only that reaction 6 be irreversible. If reaction 6 were reversible, dNMP's might bypass reaction 4.

II. Effect of Exonuclease on Fidelity: Ninio's model

In this section, approximate arguments will be presented to quantify the effect of inhibition of the 3′ → 5′ exonuclease on error rate as predicted by the Ninio (30) model. Analogous arguments using the Hopfield (9) model are considered below. In Ninio's model, there are again two stages of discrimination, one in the formation of the *E*-T-M intermediate and one in its hydrolysis. We will assume that the formation of the correct *E*-T-M intermediate is favored over the formation of the incorrect *E*-T-M intermediate by a factor f_0 and that this step is not influenced by hydrolysis rates. Ninio (30) defines, in his equation 10, a probability *P*, that a given *E*-T-M will escape hydrolysis and be incorporated. In equation 2 (Appendix I) we have formalized Ninio's general reaction scheme in a more specific way and used notations consistent with those of Hopfield (9). Our notations correspond to Ninio's as indicated: $Z' = k_2[S_2]$, $Z = k_{-2}$, $W = k_3$, $l = k_{-1}$. We will use his expression for *P* to define P_C, the probability of incorporation from a correct *E*-T-M intermediate, and P_D, the probability of incorporatioon from an incorrect intermediate. Thus,

$$P_C = \frac{Z'W}{Z'W + l_C(Z' + Z + W) + l_C^2} \tag{10}$$

and

$$P_D = \frac{Z'W}{Z'W + l_D(Z' + Z + W) + l_D^2} \tag{11}$$

Moreover,

$$f = f_0 \frac{P_D}{P_C} \tag{12}$$

But we can define P_C in another way:

$$P_C = \frac{\text{(correct incorporation)}}{\text{(correct incorporation)} + \text{(correct monophosphate generation)}} \tag{13}$$

Thus, with Mn^{2+}, $P_C = 1/(1 + 0.77)$ (Table I). Comparing equations 10 and 13, we can identify the incorporation terms with $Z'W$ and the monophosphate generation terms with those dependent upon l_C and l_C^2. We can then note

$$Z'W = 1.3 \text{ (sum of terms dependent upon } l_C \text{ and } l_C^2) \tag{14}$$

Because in Ninio's model the nature of the modification of the *E*TM intermediate in reaction 4 is not specified, it is difficult to predict exactly the relative importance of the terms in l_C and l_C^2. However, we will make the following approximate arguments. The multiplier of the l_C term in the denominator, containing as it does both Z' and W and an additional term, is larger than the largest of Z' and W. Hence, if the l_C- and l_C^2-dependent terms are 1.3-fold smaller than $Z'W$, then l_C is at least 1.3-fold smaller than the smallest of Z' and W. Hence, the term in l_C^2 is still smaller. Since l_C is smaller than the smallest of Z' and W, the greatest influence for the squared term will arise when $Z' = W$ for $Z'W$ fixed. When $Z' = W$ and Z is negligible, equation 14 becomes

$$Z'^2 \simeq 2.6\, Z' l_C + 1.3 l_C^2 \tag{15}$$

solving,

$$3.03\, l_C = Z' = W \tag{16}$$

Equations 15 and 16 are the most favorable assumptions for the Ninio model since they provide the greatest influence for the l_C^2 term. With these assumptions,

$$\frac{Z'W}{9.2} \approx \frac{(l_C\text{-dependent term})}{6.1} \approx l_C^2\text{-dependent term} \tag{17}$$

We note that the influence of the squared term is disappointingly small. To estimate f_0, we use equations 10–12, the numerical assumptions of equation 17, and the fact that $l_c/l_D = f_0$. Then

$$f = \frac{f_0 P_D}{P_C} = f_0 \frac{9.2 + 6.1 + 1}{9.2 + \left(\dfrac{6.1}{f_0}\right) + \left(\dfrac{1}{f_0^2}\right)} \tag{18}$$

or, assuming that f_0 is small enough to make the $1/f_0^2$ term in the denominator predominate,

$$f \approx 16.3\, f_0^3 \tag{19}$$

For $f = 1/4600$ (0.1 mM Mn^{2+}), equation 19 gives $f_0 = 1/42$. Substituting $f_0 = 1/42$ into the $1/f_0$ term of equation 18, we obtain

$$f = \frac{16.3\, f_0}{265 + (1/f_0^2)} \tag{20}$$

This second order of approximation yields $f_0 = 1/40$.

Now we will examine what happens to the error rate when the exonuclease rate is reduced to 60% of control (the largest inhibition observed with nucleoside monophosphates, Table V). In the exonuclease assay, there is no polymerization occurring, and the complications arising from kinetic competition between incorporation and hydrolysis are eliminated. Hence, exonuclease rates may be identified with the elementary rate constants for the hydrolysis steps, l_C and l_D. Thus, when exonuclease is reduced by a factor 0.6, the l_C- and l_D-dependent terms are reduced by a factor 0.6, and the l_C^2- and l_D^2-dependent terms are reduced by a factor $(0.6)^2 = 0.36$. Substituting into equation 18, we obtain

$$f_{60\%\text{ exonuclease rate}} = \frac{9.2 + 3.7 + 0.36}{9.2 + \left(\dfrac{3.7}{f_0}\right) + \left(\dfrac{0.36}{f_0^2}\right)} f_0 \tag{21}$$

Substituting $f_0 = 1/40$ into equation 21, we obtain an error rate of 1/2200. Hence, a 40% inhibition of exonuclease should increase the error rate from 1/4600 to 1/2200. The dependence on exonuclease rate is greater in the Ninio (30) model than the Hopfield (9) model, since the former model invokes two different hydrolysis steps, one from the *E*-T-M intermediate and one from the modified *E*-T-M* intermediate.

III. The Effect of Pyrophosphate on Error Rate: Hopfield's Model

A quantitative assessment of the effects of pyrophosphate by Hopfield's kinetic proofreading scheme can be obtained by considering the formation of the *E*TM intermediates as the rate-limiting step. Thereafter, the intermediate participates in three different competing reactions, incorporation (W), monophosphate generation (l), and reformation of the triphosphate (m).

Rate of correct incorporation

$$= \frac{\text{(Rate of formation of correct } E\text{TM intermediate)}W}{W + l_C + m} \tag{22}$$

Rate of correct monophosphate generation

$$= \frac{\text{(Rate of formation of correct } E\text{TM intermediate)}l_C}{W + l_C + m} \tag{23}$$

Rate of reformation of correct triphosphate

$$= \frac{\text{(Rate of formation of correct } E\text{TM intermediate)}m}{W + l_C + m} \tag{24}$$

By adding equations 22–24, we note that the rate of formation of the correct ETM intermediate is equal to its decomposition (the sum of incorporation, monophosphate generation, and triphosphate regeneration). Hence, our assumption that the formation of the ETM intermediate is rate limiting is equivalent to Hopfield's steady state assumption.

Similarly, three equations may be derived for the incorrect ETM intermediate, an example of which is the equation corresponding to equation 22.

$$\text{Rate of incorrect incorporation} = \frac{(\text{Rate of formation of incorrect } E\text{TM intermediate})W}{W + l_D + m} \tag{25}$$

Dividing equation 25 by equation 22, we obtain an expression for the error fraction, f, which is defined as the rate of incorrect incorporation divided by the rate of total correct incorporation when both correct and incorrect substrate are present at equal concentrations. Thus,

$$f = \frac{(\text{Rate of incorrect incorporation})}{(\text{Rate of correct incorporation})}$$

$$f = \frac{(\text{Rate of formation of incorrect } E\text{TM intermediate}) \cdot (W + l_C + m)}{(\text{Rate of formation of correct } E\text{TM intermediate}) \cdot (W + l_D + m)} \tag{26}$$

$$f = f_0 \frac{(W + l_C + m)}{(W + l_D + m)}$$

The last step comes from the assumption that the incorrect ETM intermediate is less favored by the Watson-Crick error frequency f_0.

It is important to note that m is an apparent rate constant containing the concentration of inorganic pyrophosphate as a factor. At low pyrophosphate concentration, m is close to zero, and if W is also small (which was assumed by Hopfield (9) but is not always true, see Table I), then we can neglect W and m in comparison to l_c and l_D, and equation 26 becomes

$$f = (f_0)\frac{l_C}{l_D} = f_0^2 \tag{27}$$

since $l_C/l_D = f_0$. At low pyrophosphate concentrations, the error fraction approaches $(f_0)^2$. If we add sufficient pyrophosphate to inhibit correct incorporation by 50%, then from equation 22 we note that this means increasing m from $m = 0$ to $m = W + l_C$. Substituting $m = W + l_C$ into equation 26, we obtain

$$f_{50\% \text{ inhibition}} = (f_0)\frac{2(W + l_C)}{2W + l_C + l_D} \tag{28}$$

Comparing this equation to equation 26, we observe that the numerator is twice as large. However, in the denominator l_D is 200-fold larger than l_C and 35-fold larger than W since according to the Hopfield model $l_C/l_D = f_0$. Calculations below will show that f_0 is equal to 1/200–1/800 and $W = 5.8\ l_C$. Thus, the numerator increases in proportion to inhibition by PP_i and the denominator, being dominated by l_D, remains essentially the same. At 50% inhibition, the error rate should double. In the limiting case where $m \to \infty$, the incorporation is completely inhibited (equation 22). From equation 26

$$f_{\text{total inhibition}} = \lim_{m \to \infty}\left[f_0\frac{(W + l_C + m)}{(W + l_D + m)}\right] = f_0\frac{m}{m} = f_0 \tag{29}$$

Thus, at complete inhibition, the error rate should approach f_0 which we calculate to vary between the extremes of 1/200 to 1/800 (*vide infra*). Contrary to this expectation we find that 90% inhibition with 1.6 mM pyrophosphate does not change the error rate (Table VI). A direct analysis of the effect of added pyrophosphate on the error rate using the kinetic proofreading model (9) is given below. The lack of diminished fidelity at inhibiting concentrations of pyrophosphate is not an argument against the model proposed by Ninio (30), since in this model the energy of pyrophosphate release is not directly related to fidelity.

Another method for quantitating the theoretical effect of added pyrophosphate on kinetic proofreading is by direct analysis of equation 1. Hopfield (9) derived the following exact expression for f, the error fraction, by using steady state assumptions for all activated complexes.

$$f = \frac{[l'_D(k_D + m') + m'k'_D][(k_C + m')(W + l_C) + k_C m]}{[(k_D + m')(W + l_D) + k_D m][l'_C(k_C + m') + m'k'_C]} \tag{30}$$

Let us examine the effect of pyrophosphate on error rate by noting how $1/f$ changes as a function of m.

$$d\frac{\left(\frac{1}{f}\right)}{dm} = \frac{\left(\frac{df}{dm}\right)}{f^2} = \frac{[l'_c(k_c + m') + m'k'_c]\{(k_D[k_c + m')(W + l_C) + k_c m] - k_c[(k_D + m')(W + l_D) + k_D m]\}}{[l'_D(k_D + m') + m'k'_D][(k_C + m')(W + l_C) + k_C m]^2} \tag{31}$$

In order to simplify this expression, a number of approximations can be made that are consistent with the Hopfield scheme (9). In the factors on the left-hand side of the numerator and denominator and on the right-hand side of the denominator, m' can be neglected when it appears in a sum with k_C and k_D. After expanding the factor on the right-hand side of the numerator, collecting terms, and ignoring the following terms, $k_D k_C l_C$, which is smaller than $k_D k_C l_D$, and $k_C m' W$, which is smaller than $k_C m' l_D$, equation 32 then becomes

$$\frac{d\frac{1}{f}}{dm} = \frac{[k_D m'(W + l_c) - k_C l_D(k_D + m')][l'_C k_C + m'k'_C]}{[k_C(W + l_c + m)]^2[l'_D k_D + m'k'_D]} \tag{32}$$

This equation can be further simplified. In the factors on the left-hand side of the numerator we can neglect m' in its sum with k_D. Also, the term $-k_C l_D k_D$ within this factor is by far the largest, since $l_D \gg (W + l_C)$ and $k_C \gg m'$. Thus, the factor on the left-hand side of the numerator reduces to $-k_C l_D k_D$. The factor,

$$\frac{[l'_C k_C + m'k'_C]}{[l'_D k_D + m'k'_D]} \tag{33}$$

is approximately 1 because $m' \gg l'_C$ or l'_D (the reverse of the nearly irreversible hydrolysis step). Hence, the expression is nearly equal to $m'k'_C/m'k'_D = 1$, since $k'_C = k'_D$ as discussed earlier. Equation 32, therefore, becomes

$$\frac{d\frac{1}{f}}{dm} = \frac{-k_C k_D l_D}{k_C^2(W + l_C + m)^2} = \frac{-k_D}{k_C}\frac{l_D}{(W + l_C + m)^2} = \frac{-l_D}{f_0(W + l_C + m)^2} \tag{34}$$

where we used $k_C/k_D = f_0$ in the last step. Equation 34 may be re-expressed.

$$\frac{d\frac{1}{f}}{1/f_0} = \frac{-l_D dm}{(W + l_C + m)^2} \tag{35}$$

If one lets $B = W + l_C + m$ and then integrates the change in $1/f$ from no inhibition ($m = 0$, $B = W + l_C$) to 50% inhibition [$m = W + l_C$, $B = 2(W + l_C)$] the change in error rate is equivalent to that caused by increasing pyrophosphate concentration to result in 50% inhibition of polymerization. Thus,

$$\Delta\left(\frac{1}{f}\right) = \frac{-l_D}{(2f_0)(6.9)l_C} = \frac{-1}{13.8f_0^2} = -\frac{1}{30{,}000} \tag{36}$$

since, for an error rate of 1/50,000, $f_0 = 1/640$ (see equation 38). Thus with a 50% inhibition of synthesis by pyrophosphate the error rate should approximately double from 1/60,000 to 1/30,000. Thus, the lack of increased error rate when DNA synthesis was inhibited by 90% argues against the proofreading scheme proposed by Hopfield (9).

IV. Relation between Monophosphate Generation and Fidelity

Using the same concepts, we can also consider the amount of monophosphates that would have to be generated to achieve a particular level of accuracy by the kinetic proofreading scheme. If we divide equation 23 by equation 22, we observe that the ratio of monophosphate generation to correct nucleotide incorporation is l_c/W. In Table II, this ratio was 0.17 or $W = 5.9 l_C$. Again, assuming $m \to 0$, we find, using equation 26, that

$$f = \frac{6.9 f_0 l_C}{W + l_D} \tag{37}$$

If incorrect monophosphate generation is to be favored over correct by a factor f_0, and l_C is of the order W, then l_D must be much larger than W. Hence, we may neglect W in the denominator, thus obtaining

$$f \frac{6.9 f_0 l_C}{l_D} \cong 6.9 f_0^2 \tag{38}$$

From the data in Table I, with [α-^{32}P]dGTP, $f = 1/5700$ so $f_0 \cong 1/190$. With [^{3}H]dGTP, $f = 1/21{,}000$ so that $f_0 \cong 1/380$.

Using this analysis, we can calculate that the experimentally determined generation/incorporation ratio for the incorrect nucleotide is insufficient to account for the observed error rate. Equations analogous to 22 and 23 can be used for the incorrect nucleotide. Upon division, we observe that the ratio of monophosphate generation to incorporation for the incorrect nucleotide is l_D/W. Using the value of l_C/W in Table I, which is in agreement with the studies of Deutscher and Kornberg (41), one can calculate

$$\frac{l_D}{W} = \frac{l_C}{W} \frac{l_D}{l_C} = 0.17 \frac{1}{f_0} \tag{39}$$

using values of $1/f_0$ of 1/190 or 1/380, l_D/W equals 33 or 64, respectively. Furthermore, to achieve an error rate of 1/80,000 the lowest observed, l_D/W would have to be 126. However, the data in Table I indicates that this ratio is less than 2 and, therefore, is as much as 100-fold less than that required by the Hopfield model (9). While these calculations are within the formalism of the Hopfield proofreading model (9), it is clear that the rate of monophosphate generation is insufficient to explain fidelity with the model proposed by Ninio (30) (see Appendices I and II). With a monophosphate generation-to-incorporation ratio of 2.0 for the noncomplementary nucleotide, only 2 out of every 3 potential errors can be corrected in any proofreading scheme.

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