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Nonrandom Substitution of 2-Aminopurine for Adenine during Deoxyribonucleic Acid Synthesis in Vitro†

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ABSTRACT: The incorporation of the deoxyribonucleotide of 2-aminopurine [(AP)] for deoxyadenylate into deoxyribonucleic acid (DNA) in vitro has been examined by using five highly purified DNA polymerases: calf thymus polymerase α, *Escherichia coli* polymerase I, and the polymerases induced by T4 phage mutant L56 (mutator phenotype), wild-type T4 phage, and T4 phage mutant L141 (antimutator phenotype). On a template of gapped salmon sperm DNA, the overall incorporation of (AP) relative to the incorporation of adenine decreases in this series of enzymes, in line with the increasing 3′-exonucleolytic activity associated with these polymerases. The nearest-neighbor distributions for (AP) and for adenine in the newly synthesized DNA were determined to test for potential sequence selectivity in the incorporation of (AP). In polymerizations in which d(AP)TP fully replaced dATP, the L141 polymerase, and to a lesser degree the wild type T4 polymerase, synthesized a DNA in which the distribution for (AP) was distinctly skewed compared to the nearest-neighbor distribution observed for adenine; incorporation of (AP) was relatively favored after guanine and disfavored after adenine and thymine. These sequence effects were less pronounced in syntheses in which both dATP and d(AP)TP were present. When dGTP was replaced by dITP, or dTTP by dUTP, adenine was still incorporated to the normal extent after the analogue, but the incorporation of (AP) was reduced after these analogues, which form weaker base pairs. The results indicate that incorporation of (AP) is disfavored with all polymerases tested and that a greater bias exists with those polymerases containing an active 3′-exonuclease. This bias against (AP) incorporation is alleviated after strong base pairs, and particularly following guanine, possibly due to stabilizing vertical stacking interactions.

Benzer's (Benzer, 1961) elegant analysis of the rII cistron of bacteriophage T4 demonstrated that spontaneous mutations were not distributed randomly throughout the gene and that certain loci ("hot spots") mutated at much higher frequencies than others. He suggested, at that time, that one of the factors which might contribute to this nonrandomness was the thermodynamic stability of the deoxyribonucleic acid (DNA)[1] in a particular region, and he reasoned that less stable regions

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[1] Abbreviations used: (AP), 2-aminopurine; (AP)dR, 2-aminopurine deoxyriboside; d(AP)MP and d(AP)TP, the mono- and triphosphate of (AP)dR; DNA, deoxyribonucleic acid; Tris, tris(hydroxymethyl)aminomethane.

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might be able to tolerate the incorporation of an incorrect nucleotide more readily than the more stable regions. In an earlier paper (Bessman & Reha-Krantz, 1977) we suggested a variation of that idea which took into account the properties of the 3′-exonuclease activity of these procaryotic DNA polymerases. We hypothesized that more stable regions of the DNA would be less susceptible to the editing or proofreading function of the T4-induced DNA polymerase. In this paper, we probe this hypothesis further by analyzing the substitution of the base analogue, 2-aminopurine [(AP)],[1] for adenine into the DNA synthesized with five different highly purified DNA polymerases. On the basis of nearest-neighbor analyses of the newly synthesized DNA (Josse et al., 1961), we conclude that (1) (AP) does not substitute randomly for Ade in the newly synthesized DNA, since there is a strong bias for incorporation of (AP) after Gua and a bias against incorporation of (AP) after Ade and Thy and (2) the magnitude of this bias is directly related to the relative editing capacity of the individual enzymes.

Materials and Methods

Nucleotides. Nonradioactive nucleotides were purchased from P-L Biochemicals and radioactive nucleotides from Schwarz/Mann. (AP)dR was prepared as described by Bessman et al. (1974). However, it was purified by chromatography on a column of Amberlite XAD-4 (Uematsu & Suhadolnik, 1976) instead of Dowex-1-formate, and the paper chromatography in ammonia–water was omitted. This modification was introduced because it was found that (AP)dR isolated according to the original procedure could not be phosphorylated in the next step. [α-^{32}P]dATP and [α-^{32}P]-d(AP)TP were synthesized from deoxyadenosine and (AP) deoxyriboside, respectively, according to the procedure of Symons (1974) except that in the latter case, fraction III of guanylate kinase (Oeschger & Bessman, 1966) was used to phosphorylate the d(AP)MP. 2-[6-^{3}H]Aminopurine deoxynucleoside triphosphate was synthesized as described by Bessman et al. (1974).

Nucleic Acids. Salmon sperm DNA was obtained from Worthington Biochemicals. It was partially digested by the method of Oleson & Koerner (1964). Denatured DNA refers to a preparation of 8 mM salmon sperm DNA in 0.05 N NaOH.

Enzymes. The phage-induced DNA polymerases were purified according to Lo & Bessman (1976a). For both the wild-type (T4D) and the antimutator (L141), fraction V, the homogeneous enzyme, was used. *Escherichia coli* polymerase I was fraction VII of Jovin et al. (1969), and calf thymus polymerase α was fraction 6′ of Bollum et al. (1974). A sample of highly purified calf thymus DNA polymerase kindly supplied by F. Bollum gave similar results. Spleen phosphodiesterase and micrococcal nuclease were products of Worthington, and human semen phosphomonoesterase was prepared according to Wittenberg & Kornberg (1953).

Enzyme Assays. DNA polymerase activity was measured as the conversion of acid-soluble radioactive deoxynucleoside triphosphates into an acid-insoluble product as described by Muzyczka et al. (1972). Turnover (DNA-dependent conversion of a deoxynucleoside triphosphate, dNTP, to the corresponding deoxynucleoside monophosphate, dNMP) was measured by spotting an aliquot of the standard polymerase reaction onto a poly(ethylenimine)cellulose sheet as described by Hershfield & Nossal (1972). The ultraviolet-absorbing spots were cut out and counted in a toluene-based scintillation solution. Incorporation was measured in the same incubation by removing a separate portion to measure polymerase activity by the disk assay of Bollum (1959). The percent turnover is given by

$$\% \text{ turnover} = \frac{\text{dNMP formed}}{\text{dNMP formed} + \text{dNMP incorporated}} \times 100$$

Nearest-Neighbor Analysis. All DNA subjected to nearest-neighbor analysis was synthesized under the same conditions and represented samples taken from the linear stage of the incubation. Mixtures contained the following in 0.3 mL: Tris, pH 8.8, 67 mM; $MgCl_2$, 6.7 mM; β-mercaptoethanol, 10 mM; $(NH_4)_2SO_4$, 17 mM; partially digested salmon sperm DNA, 0.83 mM; bovine serum albumin, 160 μg/mL; dCTP, dGTP, and dTTP, 50 μM; [α-^{32}P]dATP or [α-^{32}P]d(AP)TP, 50 μM, (2–5) $\times$ 10^8 cpm/μmol; and 0.01–0.2 unit of enzyme. One unit incorporates 10 nmol of radioactive nucleotide into DNA at 30 °C in 30 min. Because of the low incorporation of d(AP)MP by the antimutator enzyme, L141, this reaction volume was scaled up 5-fold in order to facilitate analysis. The samples were digested with micrococcal nuclease until >80% of the radioactivity was rendered acid soluble and then with spleen phosphodiesterase. Completeness of the digestion was monitored by treating aliquots with semen phosphomonoesterase and then measuring Norit-nonadsorbable counts. Digestion was terminated when at least 95% of the ^{32}P met this criterion. In the absence of added semen phosphatase, >98% of the radioactivity was adsorbed onto Norit indicating the absence of contaminating phosphomonoesterase activity in the nucleases employed in the digestion. Paper electrophoresis in 0.05 M sodium citrate, pH 3.5, which is usually employed at this stage to separate the nucleoside monophosphates, did not resolve d(AP)MP from dAMP. We found that descending paper chromatography in saturated ammonium sulfate–2-propanol–water (80:2:18 v/v) described by Markham & Smith (1951) clearly resolved all five nucleotides. The spots were located by radioautography and ultraviolet light, cut out, and counted in a toluene-based fluor. The sum of the counts recovered in the individual spots was >90% of those applied at the origin. The value reported for each nucleotide is given as the percent of the total nucleotides recovered from the paper. In most instances, analyses were checked by using paper electrophoresis to separate the nucleotides. In each case, the radioactivity found in the dAMP marker equaled the sum of the dAMP and d(AP)MP recovered from the paper chromatogram.

In the analysis of nearest-neighbor experiments containing other bases, different methods were sometimes required to effect resolution of the 3′-phosphates. 3′-dIMP was cleanly resolved in the above ammonium sulfate–2-propanol–water solvent, running behind 3′-dTMP and ahead of 3′-dGMP. In this system BrdUMP comigrated with dTMP, and dUMP with dCMP. However, 3′-BrdUMP and 3′-dUMP ran clearly ahead of all other nucleotides on paper electrophoresis in 0.01 M sodium borate, pH 9.2, and 3′-dTMP ran ahead of all other nucleotides on paper chromatography in 2-propanol–concentrated aqueous ammonia–water (7:2:1 v/v).

With some of the enzymes, the rate of DNA synthesis in the presence of d(AP)TP was substantially less than in the presence of dATP (see Figure 1). In order to be sure that the experimental results were not due directly to the amounts of DNA synthesized during the reaction, we also ran a control in which the two quantities of synthetic DNA were equalized by prematurely terminating the faster reaction. The enzyme used was from the antimutator L141, which showed the greatest deviation from normal. No significant difference was

Table I: Turnover of d(AP)TP by DNA Polymerases[a]

enzyme source	(a) d(AP)MP incorporated (nmol)	(b) d(AP)MP formed (nmol)	sum, a + b (nmol)	turnover, b/(a + b) × 100 (%)
$T4_{L141}$ (antimutator)	0.068	1.134	1.202	94.0
T4 (wild type)	0.306	0.462	0.768	60.2
$T4_{L56}$ (mutator)	0.556	0.631	1.187	53.2
E. coli (Pol I)	0.043	0.010	0.053	18.9
calf thymus (α)	0.206	<0.001	0.206	<0.5

[a] The procedure for measuring turnover is described under Enzyme Assays and the constituents of the incubation mixture are indicated under Nearest-Neighbor Analysis of Materials and Methods.

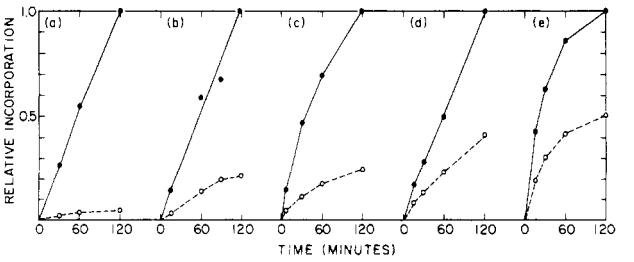


FIGURE 1: The incorporation of dATP and d(AP)TP into DNA. Reactions contained the standard reagents for nearest-neighbor analysis described under Materials and Methods with either [^{3}H]d(AP)TP or [^{3}H]dATP present at a concentration of 50 μM. Approximately 0.05 unit of each enzyme was used. The 120-min value for dATP in each incubation was set at 1.0, and all other values normalized to it in order to facilitate direct visual comparison of the graphs. The enzymes were (a) $T4D_{L141}$, (b) T4D, (c) $T4D_{L56}$, (d) *E. coli* Pol I, and (e) calf thymus polymerase α. (●) dAMP incorporation; (○) d(AP)MP incorporation.

apparent in this nearest-neighbor analysis in comparison to the standard procedure.

Results

Relative Incorporation of dATP and d(AP)TP into DNA. The kinetics of incorporation of dATP and d(AP)TP for the five enzymes used in this study are shown in Figure 1. For ease of comparison, the data have been normalized in each case by setting the 120-min value for the incorporation of dATP to 1.0 and recording all other points in each set relative to that value. In this figure, the enzymes have been arranged in the order of increasing incorporation of the analogue, d(AP)TP, relative to dATP. The values calculated for d(AP)TP/dATP at 120 min in panels a–e, respectively, are 0.05, 0.21, 0.25, 0.41, and 0.51.

Turnover of d(AP)TP. The DNA-dependent conversion of d(AP)TP to d(AP)MP (turnover of d(AP)TP) was measured for all five enzymes and is shown in Table I. The values range from 94% for the antimutator phage, L141, to undetectable in calf thymus polymerase α. The order of enzymes from lowest to highest percent turnover is the same as the order for the incorporation of d(AP)TP into DNA shown in the previous paragraph and, in respect to the phage enzymes, correlates with the exonuclease activity shown earlier by Muzyczka et al. (1972).

Nearest-Neighbor Analyses with dATP and d(AP)TP. In these experiments, [α-^{32}P]dATP or [α-^{32}P]d(AP)TP were incubated, in the absence and in the presence of equimolar unlabeled competitor, i.e., d(AP)TP and dATP, respectively, with the other three triphosphates and template–primer as described under Materials and Methods. The isolated DNA was degraded to 3′-nucleotides and analyzed for the distribution of ^{32}P among the nucleotide pairs. Routinely, four incubations were run in parallel: tubes 1 and 2 contained labeled dATP, with tube 2 containing an equal concentration (50 μM) of unlabeled d(AP)TP, and tubes 3 and 4 contained labeled d(AP)TP, with tube 4 also containing an equal concentration of unlabeled dATP. Table II summarizes the results for such nearest-neighbor experiments with all five enzymes. The results are arranged in order of decreasing nuclease to polymerase ratio, going from left to right.

If we compare the nearest-neighbor frequencies for Ade in the absence of (AP) (i.e., tube 1), it is evident that all five enzymes give approximately the same distribution for Ade, with the frequencies for CpA, ApA, GpA, and TpA registering as 22 ± 3%, 29 ± 3%, 24 ± 2%, and 24.5 ± 1.5%, respectively. Thus, the DNA synthesized in the presence of the four normal triphosphates by each of these five different enzymes is not distinguishable by this test. In contrast, striking differences are seen in the incubations containing only d(AP)TP (tube 3 experiments). These differences are exaggerated for the enzymes at the left side of Table II, and they diminish toward the right. In the case of calf thymus polymerase α the nearest-neighbor distribution observed for (AP) approximates the nearest-neighbor ratios for Ade in the control or adenine-containing DNA (tube 1). Note that the *sum* of Ap(AP) and (AP)p(AP) (24%) should be compared to the control

Table II: Nearest-Neighbor Frequencies of dAMP and d(AP)MP[a]

enzyme	L141 (amtimutator)				T4D				L56 (mutator)				*E. coli* polymerase I				calf thymus polymerase α			
tube no.	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
labeled nucleotide	Ade	Ade	(AP)	(AP)	Ade	Ade	(AP)	(AP)	Ade	Ade	(AP)	(AP)	Ade	Ade	(AP)	(AP)	Ade	Ade	(AP)	(AP)
unlabeled nucleotide		(AP)		Ade		(AP)		Ade		(AP)		Ade		(AP)		Ade		(AP)		Ade
% CpA	22	24			24	24			25	25			25	20			19	18		
% ApA	29	29			29	27			29	28			26	30			32	28		
% (AP)pA		0				1				1				4				7		
% GpA	25	24			22	23			23	23			24	23			24	22		
% TpA	24	24			25	25			23	23			26	23			25	25		
% Cp(AP)			29	22			29	22			28	23			25	27			21	19
% Ap(AP)			1	18			2	32			4	32			7	17			7	22
% (AP)p(AP)			1	1			6	0			8	0			11	2			17	9
% Gp(AP)			42	30			35	22			30	22			28	27			24	23
% Tp(AP)			28	29			29	24			30	23			29	27			32	28

[a] Incubations were carried out under standard conditions. All deoxynucleoside triphosphates were present at 50 μM. The numbers represent the ratio of the radioactivity found in the particular 3′-mononucleotide band to the total radioactivity in all five mononucleotide bands, expressed as a percentage.

Table III: Effect of Substitution of dITP for dGTP on Nearest-Neighbor Frequencies

substitution	−dGTP, +dITP (50 μM)				−dGTP, +dITP (140 μM)			
enzyme	L141 (*antimutator*)				T4D			
tube no.	1	2	3	4	1	2	3	4
labeled nucleotide	Ade	Ade	(AP)	(AP)	Ade	Ade	(AP)	(AP)
unlabeled nucleotide		(AP)		Ade		(AP)		Ade
% CpA [Cp(AP) for tubes 3 and 4]	30	30	56	37	26	26	40	32
% ApA [Ap(AP)]	35	33	4	22	29	29	5	29
% (AP)pA [(AP)p(AP)]								
% GpA [Gp(AP)]	4	3	8	6	2	2	4	2
% TpA [Tp(AP)]	25	24	29	32	24	22	36	28
% IpA [Ip(AP)]	6	10	3	3	20	21	14	9

DNA (32%), since (AP) pairs as Ade with thymine of the template strand during synthesis in vitro (Rogan & Bessman, 1970). The value of 17% for the frequency of (AP)p(AP) sequences demonstrates that calf thymus polymerase α utilizes (AP) efficiently as an analogue for adenine. The Ap(AP) sequences, which occur with a frequency of 7%, result of necessity from the condensation of d(AP)MP to original primer terminated in Ade, since the reaction mixture did not contain dATP.

With polymerase containing an increasingly active exonucleolytic function (i.e., moving from right to left in Table II), a continuous decrease in the occurrence of the (AP)p(AP) sequence is observed in tube 3 experiments, from a frequency of 11% in the case of *E. coli* polymerase I to the low value of 1% for the antimutator enzyme. The proportion of Ap(AP) sequences also decreases steadily from 7% to 1% in this series. These data indicate that an increasing nuclease to polymerase ratio of the enzyme correlates with an increasing discrimination not only against (AP)p(AP) sequences but also against Ap(AP) sequences. For if (AP)p(AP) sequences are disfavored, progressive synthesis should be aborted more often and the polymerase should more frequently revert to original primer termini, among which Ade residues are represented; on these grounds one would a priori have anticipated an *increase* in the occurrence of Ap(AP) sequences attendant on a decrease in the (AP)p(AP) frequency.

Since the nearest-neighbor frequencies in Table II are presented as percentages, a decrease in the value for Ap(AP) and (AP)p(AP) sequences is automatically accompanied by an increase in the remainder. It is obvious that in the progression from calf thymus polymerase α to the antimutator enzyme, this increase accrues disproportionately to the Gp(AP) sequence, which increases from 24% to a high value of 42% with the antimutator enzyme [31% Gp(AP) would have been observed if Gp(AP), Cp(AP), and Tp(AP) had all increased proportionately]. The frequency of Cp(AP) increases moderately in this series, from 21% to 29%, while Tp(AP) actually undergoes a moderate reduction rather than an increase, indicative of considerable bias against this sequence.

A comparison of the results in tubes 1 and 2 in Table II for the various enzymes shows that the presence of (AP) in the reaction has little influence on the nearest-neighbor distribution for Ade, except that with the exonucleolytically less active enzymes (*E. coli* Pol I, calf thymus polymerase α) a considerable portion of the ApA sequences is taken up by (AP)pA sequences in tube 2. In contrast, a comparison of tubes 3 and 4 shows that the inclusion of dATP in the incubation mixture has a profound effect on the distribution of (AP). For the L141 (antimutator) enzyme, the nearest-neighbor distribution in tube 4 is far less skewed than in tube 3; nonetheless, it is still quite different from the distribution of Ade in tubes 1 and 2. In particular, the discrimination against incorporation of (AP) after Ade is still clearly evident in this case, in which unlabeled Ade is continuously being incorporated into growing ends. However, for the other enzymes, the distribution of (AP) in tube 4 is not substantially different from the distribution of Ade in tubes 1 and 2.

Effect of dITP on Distribution of (AP) and Ade. An interesting question is whether the disproportionate incorporation of (AP) after Gua is due to some specific interaction of (AP) with a preceding base pair or to a peculiarity of the sequences involving Gua of the particular template–primer used in the incubation. Deoxyinosine (I) is an analogue of deoxyguanosine, and its triphosphate, dITP, can replace only dGTP in DNA synthesis (Bessman et al., 1958). It should therefore be incorporated into sequences only as deoxyguanosine. DNA was synthesized under standard conditions by using T4D polymerase, except that dGTP (50 μM) was replaced by dITP (140 μM). The rate of DNA synthesis, as measured by the incorporation of Ade and of (AP), was approximately the same as in the experiment with the normal complement of triphosphates. The data in Table III show that under these conditions the nearest-neighbor distributions for Ade (tubes 1 and 2) are indistinguishable from those observed in the experiment with the normal complement of triphosphates (see Table II). In particular, the sum of the GpA and IpA frequencies in the dITP experiment (23%) is identical with the frequency of GpA in the normal complement experiment. This indicates that, for this enzyme, the substitution of I for G does not perturb the tendency of Ade to be stably incorporated in the following position. However, in the experiment containing d(AP)TP for dATP (tube 3) the substitution of dGTP by dITP results in a reduction from 35% Gp(AP) to 18% for the sum of Gp(AP) and Ip(AP), with an approximately equal concomitant increase in the relative frequencies for Cp(AP) and Tp(AP). The pattern of incorporation of (AP) in competition with Ade (tube 4) is also drastically affected by the substitution of dITP for dGTP, with 22% for Gp(AP) in the normal complement experiment being reduced to 11% for the sum of Gp(AP) and Ip(AP) in the substitution experiment. The comparison of tubes 2 and 4 in the substitution experiment allows a very precise assessment of the discrimination against (AP) incorporation (as opposed to Ade incorporation) after I, since in these two tubes the DNA synthesized is indubitably the same, the incubation mixtures being *chemically* identical.

A similar experiment was carried out with the exonucleolytically more active antimutator polymerase (L141), with 50 μM dITP substituting for dGTP (Table III). Under these conditions, the Ip(AP) frequencies in tubes 3 and 4 are only 3%, and tube 3 displays a very skewed distribution, with 56% for the Cp(AP) sequence. The results for tubes 1 and 2 show that in this instance even the nearest-neighbor distributions for Ade are strongly distorted by the substitution of dITP for dGTP, the sum of GpA and IpA (~12%) being only one half of the GpA value observed in the normal complement experiment with this enzyme (25%, Table II). Nevertheless, here

Table IV: Effect of Substitution of dUTP and BrdUTP for dTTP on Nearest-Neighbor Frequencies

substitution	−dTTP, +dUTP (50 μM)				−dTTP, +BrdUTP (50 μM)			
enzyme	L141 (antimutator)				L141 (antimutator)			
tube no.	1	2	3	4	1	2	3	4
labeled nucleotide	Ade	Ade	(AP)	(AP)	Ade	Ade	(AP)	(AP)
unlabeled nucleotide		(AP)		Ade		(AP)		Ade
% CpA [Cp(AP) for tubes 3 and 4]	20	22	30	20	19	18	23	19
% ApA [Ap(AP)]	31	30	6	28	29	30	8	24
% APpA [(AP)p(AP)]								
% GpA [Gp(AP)]	29	29	47	34	26	28	42	31
% TpA [Tp(AP)]	4	3	7	5	4	4	8	5
% UpA [Up(AP)]	17	17	11	13				
% BrUpA [BrUp(AP)]					22	21	19	21

again, the comparison of tubes 2 and 4 is apposite (the identical DNA being synthesized in both cases), and it indicates a strong bias against incorporation of (AP) after I [3% Ip(AP) vs. 10% IpA].

Effects of dUTP and BrdUTP on Distribution of (AP) and Ade. In DNA synthesis with *E. coli* polymerase I, the 5′-triphosphates of deoxyuridine and 5-bromodeoxyuridine have been shown to substitute efficiently for dTTP (Bessman et al., 1958). When dTTP was replaced by 50 μM dUTP or BrdUTP, the rates of incorporation of Ade and of (AP), with the antimutator polymerase (L141), remained essentially unchanged. Table IV presents the nearest-neighbor distributions for these two cases. Replacement of dTTP by dUTP leaves the nearest-neighbor pattern for Ade almost unchanged (tubes 1 and 2, 20% for TpA plus UpA as opposed to 24% TpA in the normal complement experiment). Incorporation of (AP), however, is disfavored after U [18% Tp(AP) plus Up(AP) vs. 28% Tp(AP) in tube 3; 18% Tp(AP) plus Up(AP) vs. 29% Tp(AP) in tube 4]. In contrast, BrU very closely emulates the thymine it substitutes for in its effect on the incorporation of Ade or (AP) in the immediately following position. The frequency of incorporation of Ade after T and BrU in the substitution experiment (26%) is unchanged from the normal complement experiment (24%), as is the incorporation of (AP) in tubes 3 and 4 (27% vs. 28% and 26% vs. 29%, respectively).

Estimation of Processivity of the Polymerases. BrdUTP substitutes identically for dTTP, as evidenced by unchanged rates of incorporation for Ade and (AP) and by unperturbed nearest-neighbor distributions for these two nucleotides. Therefore, one can consider the BrU residues as equivalent to T residues, with the bromine tag serving to distinguish newly incorporated thymines from thymine residues in the original primers. This allows a simple estimation of the processivity of the enzyme, i.e., the average number of nucleotides added to a primer prior to dissociation of the polymerase. As derived in the Appendix, the processivity is approximately given by $\bar{n} = (\%\ \mathrm{TpA})_{\mathrm{norm}}/(\%\ \mathrm{TpA})_{\mathrm{BrU}}$, i.e., by the ratio of the frequencies of the TpA [or Tp(AP)] sequence in the normal complement experiment and in the experiment with BrdUTP replacing dTTP.

Applying these equations to tubes 1–4 in the BrdUTP experiment with L141 polymerase in Table IV, one obtains processivities of 6, 6, 3, and 6, respectively. For the dUTP experiment in Table IV the values are 6, 8, 4, and 6. Using the same method on the data in Table III, with T4D enzyme and dITP replacing dGTP [using $\bar{n} = (\%\ \mathrm{GpA})_{\mathrm{norm}}/(\%\ \mathrm{GpA})_{\mathrm{I}}$], one calculates processivities of 11, 11, 9, and 11, respectively. These values may be fraught with considerable error, but the following generalizations are warranted: (a) both enzymes show a low level of processivity, (b) the T4D enzyme has a higher processivity than the L141 (antimutator) enzyme, and (c) the processivity is of the same magnitude in all four tubes. This finding is of importance for the interpretation of the results because it shows that the marked skewing of the nearest-neighbor distribution observed in tube 3 experiments with the highly editing polymerases is not due to a radical change in the character of the DNA sampled; with L141 polymerase, incorporation in all cases takes place within a few nucleotide units from the original primer terminus.

For the L141 polymerase, processivity in a tube 1 incubation [with dATP, without d(AP)TP] was also determined at 10 and at 42 °C by using the BrdUTP method. In both cases, $\bar{n}$ was found to be 6.

Effect of Temperature on Nearest-Neighbor Distributions. For the L141 (antimutator) polymerase, the nearest-neighbor distributions were also determined at 10, 20, and 42 °C, under otherwise standard conditions. For all four tubes, the distributions at the various temperatures were very similar to those determined at 30 °C with this enzyme (Table II). The largest effect was a moderate increase in the Gp(AP) frequency in tube 4 experiments at the high temperature (28%, 27%, 30%, and 33%, for 10, 20, 30, and 42 °C, respectively) with a concomitant decrease in the Tp(AP) frequency (27%, 27%, 29%, and 23%).

Discussion

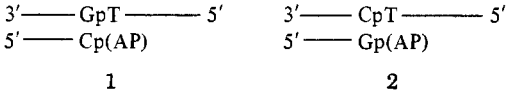
Since Freese (1959) pointed out the large variability in (AP) mutagenesis in different loci of the rII region of T4, there has been much genetic evidence which indicates that the mutagenicity of this base analogue is not directed randomly throughout the DNA. Several investigators have implicated the nucleotide sequence as an important factor in determining this variability, because large differences in (AP)-induced mutation frequencies are associated with alterations in neighboring nucleotides (Koch, 1971; Salts & Ronen, 1971; Ronen et al., 1976, 1978; Coulondre et al., 1979). Our results obtained in vitro by using purified DNA polymerases indicate that the substitution of (AP) for Ade is not a random process and that the pattern of this substitution is dependent on the particular enzyme used. The incorporation of d(AP)MP relative to dAMP (Figure 1) correlates inversely with the turnover of d(AP)TP (Table I) as seen earlier by Bessman et al. (1974) for the three phage enzymes in vitro, and it also correlates with the incorporation of (AP) into the DNA of these three phages in vivo as reported by Goodman et al. (1977). The lower turnover of *E. coli* polymerase I and the absence of turnover in calf thymus polymerase α reflect the reduced 3′-exonuclease function of the former enzyme (Kornberg, 1974) and the absence of exonuclease in the latter enzyme (Chang & Bollum, 1973). Not only are the relative amounts of d(AP)MP incorporation dependent on the enzymes but even more striking are the differences in distribution of (AP) within the newly synthesized DNA. The precision of

these measurements is indicated by the generally good agreement between tubes 1 and 2 in Table II. The very large differences in distribution of d(AP)MP in tube 3 experiments at the left of the table and their attenuation toward the right correlate with the diminution of 3′-exonuclease function in this progression. This correlation seems more than fortuitous and probably reflects the editing out of the energetically unfavorable (AP) linkages with the concomitant relative enrichment of those linkages more resistant to the exonuclease function. Since the exonuclease editing activity is related to the single-strand character of the DNA (Brutlag & Kornberg, 1972; Muzyczka et al., 1972; Lo & Bessman, 1976b), the distribution of the (AP) residues should reflect the relative stability of the helical DNA, which in turn should be related to the nucleotide sequence at the site of insertion of (AP). A striking feature of the nucleotide distribution is the very low frequency of Ap(AP) and (AP)p(AP) in the DNA synthesized by the highly editing enzyme compared to the frequency of ApA in the control DNA (Table II). The Ap(AP) arises from the addition of d(AP)TP to a primer which terminates in dAMP, and since the composition of the primer molecules was the same for all the enzymes, the comparison should be made between the frequency of ApA in the control DNA and the *sum* of Ap(AP) and (AP)p(AP) of the analogue DNA. All three of these pairs result from the copying of a TpT on the template strand. The frequency of Ap(AP) and (AP)p(AP) increases in the progression from left to right in Table II, and with calf thymus polymerase α the sum of the two pairs (24%) approaches the frequency in the control DNA (32%).

A combination of different causes may be responsible for the very low frequency of (AP)p(AP) sequences in the DNA synthesized by the efficient editors. Since these enzymes incorporate (AP) to a much lesser extent than Ade (Figure 1), the low incidence of (AP)p(AP) sequences may in part simply reflect the low probability for two rare events to occur in conjunction. However, we believe that a specific nearest-neighbor effect is also involved. It has been shown that the alternating ribopolymer poly[(AP)-U] has a considerably lower melting temperature than the adenine-containing analogue poly(A-U) (Ward et al., 1969); similarly, the homopolymer duplex poly[(AP)]·poly(U) is thermodynamically considerably less stable than poly(A)·poly(U) (Janion & Shugar, 1973). Clayton et al. (1979) have calculated the (AP):T base pair to be less stable than the A:T pair by 1.1 kcal/mol. The discrimination against overall incorporation of (AP) is related to the high turnover for this nucleotide (Table I), which in turn is occasioned by the weakness of the (AP):T pair at the growing point; it stands to reason that the exonucleolytic action of the highly editing enzymes will be particularly pronounced when the (AP):T pair at the growing point is further destabilized by an immediately preceding weak (AP):T base pair. However, the impediment to incorporation of (AP) after a weak base pair is documented with less ambiguity in the substitution experiments (Tables III and IV), where the probe [(AP)] is *not* identical with the perturbing nucleotide (I, U, or BrU).

The discrimination against Ap(AP) sequences manifested by the L141 polymerase in the tube 3 experiment in Table II is certainly significant, given the low degree of processivity of this enzyme, which assures frequent return to primer termini. From the BrdUTP experiments in Table IV we estimated the processivities for the L141 (antimutator) enzyme and the T4D enzyme in tube 3 incubations to be 3 and 9, respectively. Therefore, (AP) will add to original termini in one-third and one-ninth of the cases, respectively. Approximately one-fourth of these termini are constituted by Ade. Thus, if there is no bias against Ap(AP) sequences, they should make up $^1/_{12}$ (8%) of all Xp(AP) sequences in the case of L141 polymerase and $^1/_{36}$ (3%) in the case of the T4D polymerase. Clearly, this standard is not reached with the L141 enzyme [Ap(AP) = 1%], but it is approached in the case of the T4D enzyme [Ap(AP) = 2%]. The bias against Ap(AP) synthesis by the L141 (antimutator) polymerase is also evident in tube 4 (Table II), where at any given A site the enzyme has a choice between Ade and (AP): we find 18% Ap(AP) [plus 1% (AP)p(AP)] as opposed to 29% ApA in tube 2.

The other striking result in Table II is the increasing trend for Gp(AP) sequences in tube 3 experiments, as one progresses from calf thymus polymerase α to the L141 (antimutator) enzyme. Why is Gp(AP) enriched by the highly editing polymerases? If the incorporation of (AP) were favored due to stabilization at the growing point by a preceding G:C base pair, it might be expected that *both* Gp(AP) and Cp(AP) would be favored. In fact, compared to the results with the calf thymus enzyme, the tube 3 experiments with the highly editing polymerases indicated a moderate bias against incorporation of (AP) after Thy, a very slight bias in favor of the Cp(AP) sequence, and a pronounced preference for the Gp(AP) sequence. In examining structures **1** and **2**, it is obvious that



1 **2**

the thermodynamic stability of the newly incorporated (AP) nucleotide is defined primarily by hydrogen bonding to Thy of the template strand (an effect which is identical in the two structures) and by vertical stacking interactions with the preceding base, Cyt in structure **1** and Gua in structure **2**. It is quite possible that the vertical stacking interactions are stronger in Gp(AP) than in Cp(AP), resulting in greater stabilization of the former sequence and thus in its preponderance in the newly synthesized DNA. This interpretation in terms of favorable G–(AP) stacking is necessarily hypothetical at present, due to the paucity of physical studies on polynucleotides which contain (AP). There are precedents for our observation that the degree of misincorporation of (AP) can differ considerably, depending on whether it follows one base or the other of a given base pair: on both poly(dA)·(dT) and poly(dG)·(dC) as template–primers, T4 polymerase misinserts nucleotides largely after the purines, not the pyrimidines (Gillin & Nossal, 1976; Topal et al., 1980).

The fact that the nearest-neighbor preferences for incorporation of (AP) are more pronounced in tube 3 experiments [where A sites can be occupied only by (AP)] than in tube 4 incubations [where Ade and (AP) compete at each A site] may appear incongruous at first sight. One might have expected the nearest-neighbor distributions for (AP) in tube 3 experiments to be identical with the nearest-neighbor ratios for Ade in tube 1 incubations, indicating that in the absence of competition by Ade, the analogue, (AP), substitutes equally for it at all sites. This situation is, indeed, approached in polymerizations with the the least editing enzyme, calf thymus polymerase α. One must keep in mind, however, that, even in the absence of competing dATP, the enzyme has several options at any given A site: it can dissociate from the particular primer *without incorporating* (AP), it can abort sequential synthesis *after incorporating* (AP), or it can *incorporate* (AP) and *continue synthesis* on the same primer, downstream from the newly incorporated (AP). With the L141 (antimutator) enzyme, the first alternative, dissociation

without incorporation of (AP), is heavily favored. This is apparent from the fact that (AP) is incorporated at a much lower rate than Ade (Figure 1, panel a), even though the processivity is low in both experiments: with dATP, the L141 polymerase will incorporate about one dAMP and dissociate then or soon thereafter, but with d(AP)TP the enzyme is in difficulty at the first A site it encounters, and it will in most instances dissociate without stably incorporating d(AP)MP at that site. Because of this strong bias against incorporation of (AP), the selectivity is particularly high in this instance, giving rise to pronounced biases for and against particular nearest neighbors. It is important to realize that this high level of selectivity is not a direct result of the low processivity; in the d(AP)TP experiment (tube 3) with calf thymus polymerase α, the nearest-neighbor ratios are close to normal, even including (AP)p(AP) sequences, in spite of the fact that (as pointed out below) the processivity is rather low.

For L141 polymerase, the nearest-neighbor biases for (AP) are much attenuated in tube 4, compared to tube 3. The reason may lie in the following: given the already severe bias against (AP) incorporation in tube 3 with this highly editing enzyme, the presence of dATP in the tube 4 experiment does not appreciably raise the criterion for incorporation of (AP). In fact, the rate of incorporation of (AP) in tube 4 was fully two-thirds the rate in tube 3. Under these circumstances, the main effect of added dATP on the distribution of (AP) may be protection of (AP) incorporated at less select positions against exonucleolytic action, by further synthesis downstream from the (AP). Such more extended synthesis will more readily occur in tube 4, which contains the full normal complement of triphosphates, and the processivity was indeed found to be higher in tube 4 (and in tube 2) than in tube 3. The higher degree of processivity of the T4 enzyme, allowing for more extended synthesis, may be responsible for the fact that with this enzyme the nearest-neighbor distribution for (AP) in tube 4 already appears dampened out to a normal pattern.

For the highly editing phage polymerases, peelback (i.e., sequential exonucleolytic action) is a real possibility. Thus, the susceptibility of an (AP) residue to excision is a function not only of the preceding but also of the following sequence. For this reason, the results in tube 3, where incorporation after (AP) occurs only rarely, are more apt than the results in tube 4 to focus on the importance of the immediately preceding base for (AP) incorporation.

The effect of dATP on the incorporation of d(AP)TP is complex. Since (AP) is an analogue of Ade (Freese, 1959) and is incorporated in DNA in vitro in place of Ade (Rogan & Bessman, 1970), dATP and d(AP)TP should compete for the same sites during synthesis. Clayton et al. (1979) have reported that the K_M for d(AP)TP is 6-fold greater than for dATP for the three phage enzymes used in our study, and so it would be expected that dATP would have a strong inhibitory effect on the incorporation of d(AP)TP into DNA. This result is indeed observed at elevated levels of dATP, as seen in Figure 2. However, this figure shows that the effect of dATP on the incorporation of d(AP)TP is ambivalent, as was also noted by Clayton et al. (1979) in a different context. At low levels of dATP there is actually a stimulation of (AP) incorporation, which reaches a maximum at a ratio of dATP/d(AP)TP of 0.1. Presumably, dATP allows DNA synthesis to proceed past runs of Thy on the template which are roadblocks to the incorporation of (AP) [as evidenced by the low (AP)p(AP) frequency], and dATP may also stabilize incorporated (AP) by participating in polymerization downstream, thus counteracting peelback. This is an interesting variation in which

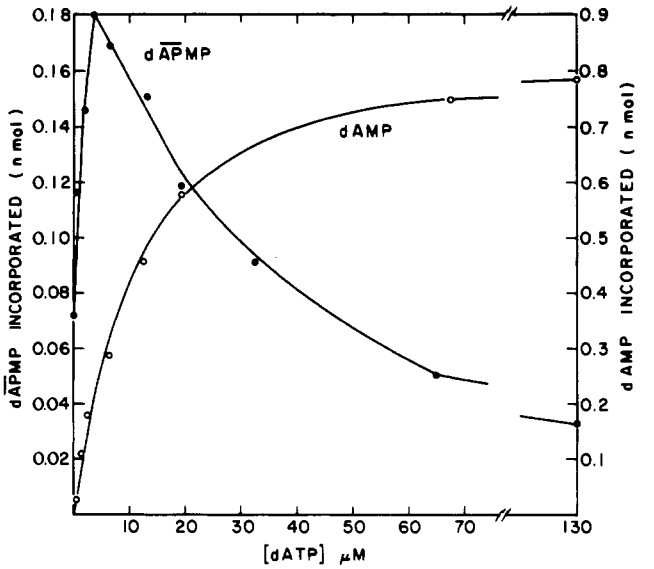


FIGURE 2: Effect of dATP on the incorporation of d(AP)TP into DNA by T4 DNA polymerase. The incubation mixture contained the following in 0.150 mL: Tris, pH 8.8, 67 mM; $MgCl_2$, 6.7 mM; β-mercaptoethanol, 10 mM; $(NH_4)_2SO_4$, 17 mM; denatured DNA, 1 mM; bovine serum albumin, 160 μg/mL; dCTP, dGTP, and dTTP, 30 μM; [^{3}H]dAPTP, 30 μM, 1×10^8 cpm/μmol; [^{14}C]dATP, as indicated, 1.8×10^7 cpm/μmol; and 0.05 unit of T4 DNA polymerase. After 20 min at 37 °C the aliquots were plated on glass fiber disks, precipitated with trichloroacetic acid, washed, and counted.

a potential competitive inhibitor actually stimulates the reaction. It is another aspect of the influence of the pool size of the various triphosphates in determining misincorporation rates as discussed by Galas & Branscomb (1978), Clayton et al. (1979), Fersht (1979), and Hopkins & Goodman (1980).

The influence of incorporated hypoxanthine on the nearest-neighbor distributions is informative because it shows that although hypoxanthine can specifically replace guanine in DNA (Bessman et al., 1958), it interacts with other bases in a very different manner. Hypoxanthine was chosen for this experiment because the intent was to change a strong base pair G:C to a very weak base pair I:C without changing the potential sites for entry of (AP). Poly(dG)·poly(dC) has a T_m over 50 °C higher than poly(dI)·poly(dC) (Inman & Baldwin, 1964). The data in Table III support the view that the weakened structure at the I:C site is very unfavorable to the stable incorporation of (AP). For the strongly editing L141 polymerase, even Ade does not incorporate well after I. The weakened DNA structure would be especially susceptible to 3′-exonuclease activity. In fact, DNA which contains I is more rapidly hydrolyzed by the editing function of T4 polymerase (M. J. Bessman, unpublished observation). The pronounced preponderance of Cp(AP) sequences (56%) over Tp(AP) sequences (29%) in the tube 3 experiment with the antimutator polymerase is probably an expression of the aforementioned intrinsically superior ability of Cyt, compared to Thy, to stabilize the incorporation of (AP) at an immediately following position, accentuated in this instance by the extreme stringency of the polymerization conditions: the use of the highly editing polymerase and the substitution of *two* bases, Ade and Gua, by weakly base pairing analogues, (AP) and I. It is significant that in the corresponding experiment with the exonucleolytically less active T4 polymerase, the Cp(AP) and Tp(AP) frequencies in tube 3 (40% and 36%, respectively), while still showing a slight preponderance of the Cp(AP) sequence, are approaching the relative ratio of unity observed when dGTP is not substituted by dITP (29% and 29%, Table II, T4D, tube 3).

Compared to the substitution of G by I, replacement of T by U or BrU causes a much lower perturbation of helix stability. The melting temperatures reported by Ward et al. (1969) for ribopolymers containing alternating (AP) and thymine derivatives are particularly germane to this topic: 28 °C for poly[(AP)-U)], 42 °C for poly[(AP)-T)], and 51 °C for poly[(AP)-BrU)], all at 0.001 M Na^+. Now, even with the highly editing L141 polymerase, the relatively minor destabilization caused by substitution of U for T hardly affects the nearest-neighbor distribution for Ade but still strongly militates against incorporation of (AP) after U (Table IV). In contrast, after the helix-*stabilizing* substitution of BrU for T, the nearest-neighbor distributions for *both* Ade and (AP) remain essentially unchanged (Table IV). Taken in conjunction, the data for the nucleotide substitution experiments in Tables III and IV demonstrate that the incorporation of (AP), either alone or in competition with Ade, after a weak base pair is more strongly disfavored with decreasing thermodynamic stability of that base pair and with increasing exonucleolytic activity of the polymerase. The data thus bear out the marked influence of the stability of the immediately preceding DNA region on the propensity for misincorporation of (AP) for Ade at a given site.

The comparison of TpA frequencies in polymerization with the normal complement of triphosphates with the TpA frequencies obtained when dTTP is substituted by an efficient analogue provides a simple means to determine the processivity of the polymerase. For the L141 (antimutator) and the T4D polymerases, this method gave processivities of 6 and 11, respectively. For the calf thymus enzyme we can estimate, less rigorously, a value of $\bar{n}$ of ~4.6 in the polymerization with d(AP)TP [from 32% ApA in tube 1 compared to 7% Ap(AP) in tube 3]; the processivity with dATP will probably be somewhat higher. For two of these enzymes, we can compare our estimated processivities, at 30 °C, with the values obtained by Das & Fujimura (1979) using a different method, with poly(dA)·oligo(dT) as template–primer: for the T4-induced DNA polymerase they determined the processivity to be 3 at 25 °C and 12 at 37 °C, while for calf thymus polymerase α the processivities were 11 and 8 at these temperatures. With due regard to the differences in the templates and in the temperatures involved in this comparison, we can state that the new method proposed here for the determination of processivities and the established procedure of Das & Fujimura give similar results. The new procedure should be a useful complement to other methods for determining processivities, e.g., the cycling-time perturbation method of Bambara et al. (1978) or the template challenge method of McClure & Jovin (1975).

For both Ade and (AP), the nearest-neighbor distribution in the DNA synthesized by the L141 polymerase was found to be hardly changed over the temperature span from 10 to 42 °C. This result is understandable in view of the fact that the difference in stability between various DNAs—or different base pairs—is largely caloric in nature, the differences in the entropic term generally being minor (Klump & Ackermann, 1971). Since it is in the latter term that the temperature dependence of the free energy largely resides [the enthalpy of formation of polynucleotide duplexes is relatively insensitive to temperature changes, see e.g., Privalov et al. (1969)], the differences in stability between various DNAs or different duplex regions is not a sensitive function of temperature. Thus, a preceding Gua residue is expected to impart to a newly inserted (AP) approximately the same special stabilizing $\Delta(\Delta G)$ contribution at 42 °C as it does at 10 °C.

In the interpretation of these results, several factors must be considered. First, the data reveal "hot spots" and "cold spots" of *incorporation* of (AP) but say nothing about the *specific mutagenicity* of the incorporated (AP). If the (AP) is incorporated selectively because it forms a more stable configuration with neighboring nucleotides, its mispairing capacity upon which its mutagenicity depends (Freese, 1959) could be enhanced or diminished. Koch (1971) inferred from one study that an incorporated (AP) mispairs with Cyt 6–20 times more frequently if the (AP) is preceded by a G:C rather than an A:T pair. The mismatch of (AP) with Cyt is thought to involve the imino tautomer of (AP) (Topal & Fresco, 1976). Since the tautomerism of a mismatched base in double-helical nucleic acid appears to be strongly modulated by intrastrand stacking interactions (Fresco et al., 1980), certain local base sequences may be most favorable for mispairing of (AP) with Cyt. These sequences, however, are not necessarily those most conducive to incorporation of (AP) for Ade in the first place. Second, our data say nothing about "errors in incorporation", that is, the incorporation of (AP) opposite a template Cyt which presumably causes G:C → A:T transitions. Third, although there appears to be a strong correlation between editing capacity and bias in (AP) incorporation, the nexus between the two has not been proved. There have been several reports of nonuniform incorporation of analogues by RNA polymerase which presumably has no editing function (Goldberg & Rabinowitz, 1961; Kahan & Hurwitz, 1962; Novogrodsky et al., 1966; Slapikoff & Berg, 1967). The differences in the properties and mechanisms of synthesis by these two classes of enzymes make it difficult to relate these observations.

The nearest-neighbor analysis has demonstrated that (AP) is not distributed randomly for Ade in DNA synthesized in vitro by several enzymes. Our intent was to assess the generalized influence of the *immediately* preceding base on the propensity for stable incorporation of (AP) at A sites, sampling a very large number of local sequences. This assures the general validity of our conclusions; at the same time, the averaging involved in subsuming a large number of different local sequences under the heading of one nearest neighbor causes the sequence effects observed at the nearest-neighbor level to be generally subtle, except for such striking results as the bias against Ap(AP) sequences and for Gp(AP) sequences shown by the L141 polymerase and the discrimination against (AP) after I and U. Since misincorporation of (AP) for Ade is dependent on local duplex stability (as shown in this paper) and since this stability is a function not only of the nearest-neighbor but also of the preceding sequence of several nucleotides, we expect the degree of incorporation of (AP) at discrete sites to evince much wider fluctuations than those manifested by the nearest-neighbor criterion. Experimental procedures to quantitate incorporation of (AP) for Ade at defined sites in known DNA sequences are at present being developed.

Appendix

The following is a derivation of the formula for estimation of the degree of processivity of the enzyme from the frequency of TpA sequences in polymerization with the normal complement of triphosphates and the TpA frequency in polymerization with BrdUTP substituting for dTTP. The degree of processivity (average length of chain extension) is

$$\bar{n} = \frac{\text{no. of all incorporations}}{\text{no. of all incorporations at original ends}}$$

By use of A as a probe, representative of all nucleotides

$$\bar{n} = \frac{\text{no. of all XpA's}}{\text{no. of all XpA's at original ends}}$$

In the case of the BrdUTP substitution experiment, this ratio is

$$\bar{n} = \frac{\%\ \text{BrUpA} + \%\ \text{TpA} + \%\ \text{ApA} + \%\ \text{GpA} + \%\ \text{CpA}}{\%\ \text{TpA} + (\%\ \text{ApA})_{\text{ends}} + (\%\ \text{GpA})_{\text{ends}} + (\%\ \text{CpA})_{\text{ends}}}$$

Here, $(\%\ \text{XpA})_{\text{ends}}$ denotes that fraction of A incorporation occurring at original primer terminated in X. The numerator is, of course, unity. In the denominator we factor out % TpA:

$$\bar{n} = 1/\%\ \text{TpA}\left[1 + \frac{(\%\ \text{ApA})_{\text{ends}}}{\%\text{TpA}} + \frac{(\%\ \text{GpA})_{\text{ends}}}{\%\ \text{TpA}} + \frac{(\%\ \text{CpA})_{\text{ends}}}{\%\ \text{TpA}}\right]$$

We now equate the terms $(\%\ \text{XpA})_{\text{ends}}/\%\ \text{TpA}$ with the corresponding ratios $(\%\ \text{XpA})_{\text{norm}}/(\%\ \text{TpA})_{\text{norm}}$, measured in the normal complement experiment; i.e., we take the nearest-neighbor distribution determined for A in sequential synthesis in the normal complement experiment to be identical with the nearest-neighbor frequency for A at original ends. This presupposes that there is no bias in the identity of the original termini, i.e., the base distribution among original ends is the same as the overall base distribution in the DNA:

$$\bar{n} = 1/\%\ \text{TpA}\left[1 + \frac{(\%\ \text{ApA})_{\text{norm}}}{(\%\ \text{TpA})_{\text{norm}}} + \frac{(\%\ \text{GpA})_{\text{norm}}}{(\%\ \text{TpA})_{\text{norm}}} + \frac{(\%\ \text{CpA})_{\text{norm}}}{(\%\ \text{TpA})_{\text{norm}}}\right]$$

$$\bar{n} = 1/\%\ \text{TpA}\frac{(\%\ \text{TpA})_{\text{norm}}}{(\%\ \text{TpA})_{\text{norm}}} + \frac{(\%\ \text{ApA})_{\text{norm}}}{(\%\ \text{TpA})_{\text{norm}}} + \frac{(\%\ \text{GpA})_{\text{norm}})}{(\%\ \text{TpA})_{\text{norm}}} + \frac{(\%\ \text{CpA})_{\text{norm}}}{(\%\ \text{TpA})_{\text{norm}}}$$

The sum over the four % XpA's for the normal complement experiment is unity

$$\bar{n} = \frac{1}{\%\ \text{TpA}\dfrac{1}{(\%\ \text{TpA})_{\text{norm}}}} = \frac{(\%\ \text{TpA})_{\text{norm}}}{\%\ \text{TpA}}$$

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Subfractionation of Malignant Variants of Metastatic Murine Lymphosarcoma Cells by Countercurrent Distribution in Two-Polymer Aqueous Phases†

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ABSTRACT: The low metastatic murine lymphosarcoma parental cell line RAW117-P and a high metastatic subline (RAW117-H10), selected sequentially 10 times for liver colonization, were grown in suspension and subjected to countercurrent distribution (CCD) in a dextran–poly(ethylene glycol) aqueous phase system having an electrostatic potential difference between the phases. Both the RAW117-P and the RAW117-H10 sublines gave broad distribution curves, with the mean partition coefficient of RAW117-H10 cells being higher than the mean partition coefficient of RAW117-P cells. RAW117-P cells were kept in culture over a 2-month period during which time the mean partition coefficients increased. Concomitant with their in vivo drift in partition coefficients, the RAW117-P cells also displayed increasing heterogeneity as evidenced by the appearance of broader and even multipeaked CCD curves. However, the mean partition coefficients always remained lower than that of the RAW117-H10 subline. Cells from different cavities along the extraction train following CCD of the RAW117-P line were placed into tissue culture, allowed to double overnight, and assayed in vivo by injection of 5×10^3 cells intravenously into BALB/c mice. After 20 days, the RAW117-P cells which had the higher partition coefficients (corresponding more closely to the higher mean partition coefficient of the high metastatic RAW117-H10 subline) formed significantly more liver tumor colonies than did the RAW117-P cells with lower partition coefficients. Analysis of surface proteins on the CCD-subfractionated RAW117-P cells by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and staining with ^{125}I-labeled concanavalin A indicated that the cell-surface glycoproteins of the more metastatic cell subpopulations had a decrease in an ~70 000 molecular weight component similar to the highly metastatic subline RAW117-H10. These results suggested that malignant cell variants exist in the parental RAW117-P population and that they can be separated from the majority of cells of low malignant potential by countercurrent distribution.

Tumor metastasis involves a complex series of sequential steps whereby malignant cells spread from primary to near and distant secondary sites where they arrest, invade, and proliferate to form new tumor foci (Fidler et al., 1978; Poste & Fidler, 1980; Fidler & Nicolson, 1981). This phenomenon appears to be the end result of several highly selective steps in which fewer and fewer tumor cells survive ultimately to form secondary growth (Poste & Fidler, 1980; Fidler & Nicolson, 1981). The concept suggests that the cells capable of metastasis represent a minor subpopulation of cells comprising the primary tumor (Fidler & Kripke, 1977; Kripke et al., 1978; Nicolson et al., 1978) and that these highly malignant tumor cells have unique characteristics important in determining their metastatic properties (Nicolson et al., 1980; Fidler & Nicolson, 1981).

Several animal tumor models have been developed to establish the tumor cell and host properties important in metastasis (Poste & Fidler, 1980; Fidler & Nicolson, 1981; Nicolson et al., 1978, 1980). One such model utilizes a murine lymphosarcoma tumor line called RAW117 that is capable of forming solid tumor nodules in liver, lungs, spleen, and lymph nodes of injected mice. We have used the parental lyumphosarcoma line RAW117-P to select sublines with enhanced potential to colonize liver. After ten sequential selections for liver colonization, subline RAW117-H10 was obtained which forms 200–250 times more gross liver tumor nodules compared to line RAW117-P within approximately 2 weeks after injection intravenously or subcutaneously and displays enhanced malignancy when assayed by time of host death (Brunson & Nicolson, 1978; Reading et al., 1980b; Nicolson et al., 1980).

The RAW117 sublines with enhanced malignant characteristics have cell-surface alterations that correlate with their biologic properties (Reading et al., 1980b). Utilizing sequential selection procedures based upon lack of cell adherence to immobilized lectins, such as concanavalin A (Con A),[1] we have also obtained RAW117 sublines that possess modified mal-

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[1] Abbreviations used: Con A, concanavalin A; CCD, countercurrent distribution; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; FBS, fetal bovine serum; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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