Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 302–306, January 1982
Biochemistry

Are DNA precursors concentrated at replication sites?

(DNA replication rates/replication proteins/mutation rates/DNA precursor pools/functional compartmentation)

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Communicated by Peter H. von Hippel, September 21, 1981

ABSTRACT **We have asked whether the effective concentrations of deoxyribonucleoside 5'-triphosphates (dNTPs) at sites of DNA replication *in vivo* might be higher than the concentrations of dNTPs averaged over the entire cell volume. The approach involved determination of the dependence of DNA replication rate upon thymidine triphosphate concentration, both *in vivo* and in an *in vitro* system that closely approximates the intracellular replication apparatus. In T4 phage-infected *Escherichia coli* maximal rates of DNA synthesis were attained with dTTP pools of approximately 1.2×10^5 molecules per cell, corresponding to an average intracellular concentration of about 65 μM. When DNA synthesis was measured in the T4 purified protein system [Sinha, N. K., Morris, C. F. & Alberts, B. M. (1980) *J. Biol. Chem.* 255 4290–4303], maximal rates were observed at dTTP concentrations of 200–240 μM. This represents a minimal estimate, therefore, of dTTP concentration at replication sites and suggests that at least a 3- to 4-fold concentration gradient exists near these sites. We discuss why such concentration gradients might be needed and how they might be generated. We also discuss the implications of these results for understanding the relationship between intracellular dNTP pools and mutation rates. A by-product of our study was the finding that exogenous thymidine is used for T4 DNA synthesis in preference to endogenous pathways to thymine nucleotides; at high thymidine concentrations *in vivo* the endogenous pathways can be completely bypassed.**

What are the effective concentrations of DNA precursors at replication sites? This question is of interest for several reasons: (*i*) The rate of DNA synthesis *in vivo* seems not to be limited by substrate availability (1), yet *in vitro* systems for DNA synthesis require concentrations of deoxyribonucleoside 5'-triphosphates (dNTPs) that are much higher than average intracellular concentrations as estimated from pool measurements. (*ii*) DNA precursors are compartmentalized, and several lines of investigation indicate that only a small fraction of the total dNTP in a cell is readily available to participate in DNA replication (2–5). (*iii*) The compartmentation may be brought about by the action of specific dNTP-synthesizing multienzyme complexes, which behave as though juxtaposed with the replication apparatus (6–11); such complexes seem able, *in vitro*, to maintain concentration gradients of deoxyribonucleotide intermediates (7). (*iv*) DNA precursor concentrations represent a determinant of the fidelity of DNA replication. Deoxyribonucleotide pool imbalances, generated either in cell-free DNA replication systems or *in vivo*, lead to increased spontaneous mutation rates (12–22), presumably by increasing the frequency of mispairings during replication. Knowledge of dNTP concentrations at replication sites is important for understanding the extent to which natural mutation rates are governed by precursor pool effects.

Because of the compartmentation alluded to above, direct determination of effective dNTP levels is difficult, if not impossible. This paper represents a first attempt to estimate, by indirect methods, the effective concentration of thymidine triphosphate (dTTP) at sites of DNA replication for bacteriophage T4 *in vivo*.

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MATERIALS AND METHODS

DNA synthesis *in vitro* was measured by use of the T4 phage purified protein system, as described by Sinha *et al.* (22). For measurement of DNA synthesis *in vivo*, thymidine incorporation into DNA was determined as described by Mathews (1). Thymine nucleotides were extracted and quantitated as described (1).

Experiments on DNA synthesis and thymine nucleotide pool measurements used *Escherichia coli* 201, a low-thymine-requiring strain of *E. coli* B, originally obtained from G. R. Greenberg. Phage strains used included T4D (wild-type); T4 *td8nd28* [*td*$^-$*denA*$^-$, deficient in thymidylate synthase induction and host cell DNA breakdown (23)]; T4 *td8ts*L159 [*td*$^-$*45*^{ts}, deficient in thymidylate synthase induction and temperature-sensitive in gene *45*, essential for DNA replication (1)]; and T4 *td8nd28ts*L159. This triple mutant was constructed by standard genetic methods.

RESULTS

The rationale for this investigation is as follows. The T4 phage purified seven-protein (p32, p41, p43, p44, p45, p61, and p62) system for DNA synthesis *in vitro* catalyzes DNA chain elongation at rates approaching those seen *in vivo* (22). The accuracy of base selection in this system is close to that achieved in normal DNA replication *in vivo* (17, 19). In several other ways this system closely simulates the behavior of T4 DNA replication forks in intact cells. If one can establish that dNTP levels are indeed saturating at replication sites *in vivo*, then the minimal dNTP concentrations that give maximal rates of DNA synthesis *in vitro* should represent the minimal effective concentrations at replication sites in whole cells.

When discussing DNA replication *in vivo*, it is probably more appropriate to speak of activities than concentrations, because of the unknown effects of environmental factors at replication sites, such as hydrophobicity or viscosity of the intracellular milieu. In any event, it is desirable to be able to measure accurately the rate of DNA synthesis *in vivo*, and to determine its dependence both upon exogenous thymidine concentration and upon internal thymine nucleotide pools. This allows both an estimation of the extent to which dNTPs are concentrated at replication sites (from the difference between average intracellular concentration and estimated effective concentration) and a determination of whether the replication apparatus is normally saturated with dNTPs *in vivo*.

In T4 phage-infected *E. coli* there are three metabolic routes to endogenous formation of thymine nucleotides: host cell thymidylate synthase, phage-coded thymidylate synthase, and

phage-induced bacterial DNA breakdown (24). Because each of these pathways can be inactivated by mutation, one can render phage DNA synthesis wholly dependent upon exogenous thymidine, and hence, one can accurately determine rates of DNA synthesis from rates of thymidine incorporation into DNA. Because of these technical advantages, the current study is focused upon thymine nucleotide metabolism. In principle, however, this approach could be extended to other nucleotides.

Thymidine Triphosphate Concentration Dependence of T4 DNA Synthesis In Vitro. When the concentration of an equimolar mixture of dNTPs is varied, the seven-protein system for T4 DNA synthesis *in vitro* reaches saturation at about 200 μM each. When dTTP alone was varied, saturation was reached at 220 μM, with a decline at higher levels (Fig. 1). Similar results were seen in an experiment identical to this except for the substitution of 5-hydroxymethyl-dCTP for dCTP (data not shown). Another series of experiments tested the effects of alterations that might more faithfully reflect conditions *in vivo*: addition of inert protein (bovine serum albumin), or increases in salt concentration. In all cases dTTP levels of more than 200 μM were required to reach saturation (data not shown).

Aspects of the Kinetics of T4 DNA Synthesis. To optimize labeling conditions we wanted to learn the extent to which host cell DNA breakdown contributes thymine nucleotides to phage DNA synthesis when other precursor supplies are adequate. By comparing thymidine incorporation rates under conditions in which both host and phage thymidylate synthases are inactivated with corresponding data from the system in which host DNA breakdown is blocked as well, the contribution of the breakdown pathway can be estimated. This experiment was carried out at 100 μM exogenous thymidine, a relatively high concentration. No difference in rates of thymidine incorporation was seen when we compared T4 *td8* with T4 *td8nd28* (breakdown-positive with breakdown-negative, respectively; data not shown). This indicates that under these conditions thymine nucleotides derived from host cell DNA degradation are not used for phage DNA synthesis. To our surprise, even wild-type T4 phage incorporates exogenous thymidine into its DNA at the same rate as T4 *td8nd28*, at least during the first 40 min after infection. Preferential utilization of exogenous thymidine for DNA synthesis has been reported in other biological systems (25, 26) but not, to our knowledge, in T4 phage-infected bacteria. We have recently obtained evidence for T4 thymidine kinase as an element of the deoxyribonucleotide-synthesizing multienzyme complex of T4 (unpublished results). The findings reported here suggest that the enzyme thymidine kinase might be functionally linked, as well as physically associated, with other enzymes of DNA precursor synthesis. As expected, the ability of exogenous thymidine to compete with endogenous pathways decreases as the concentration of exogenous thymidine is lowered (Fig. 2). The data show that at thymidine concentrations of 80 μM or higher thymidine incorporation rates can be used as a valid measure of DNA synthesis in this system even in the presence of intact endogenous synthetic pathways.

The Replication Apparatus Is Saturated with dTTP in Intact Cells. One way to determine whether replication sites are saturated with precursors *in vivo* is to expand the precursor pools and ask whether this leads to increased rates of DNA synthesis. In a previous study, one of us (1) found that thymidine nucleotide pools expand rapidly when DNA synthesis is reversibly blocked by use of a temperature upshift and a temperature-sensitive mutation in a gene essential to DNA replication. The phage mutant used was T4 *td8ts*L159 (dTMP synthetase negative, 45^{ts}). When DNA synthesis was restored by a temperature downshift after such a pool expansion, little or no increase in rate of thymidine incorporation was seen.

Because our earlier work was done under conditions in which the host cell DNA breakdown pathway for nucleotide production was active, we did a comparable experiment here with T4 *td8nd28ts*L159, in which the *denA* mutation (*nd28*) blocks host cell DNA breakdown. Fig. 3 shows that a culture infected at

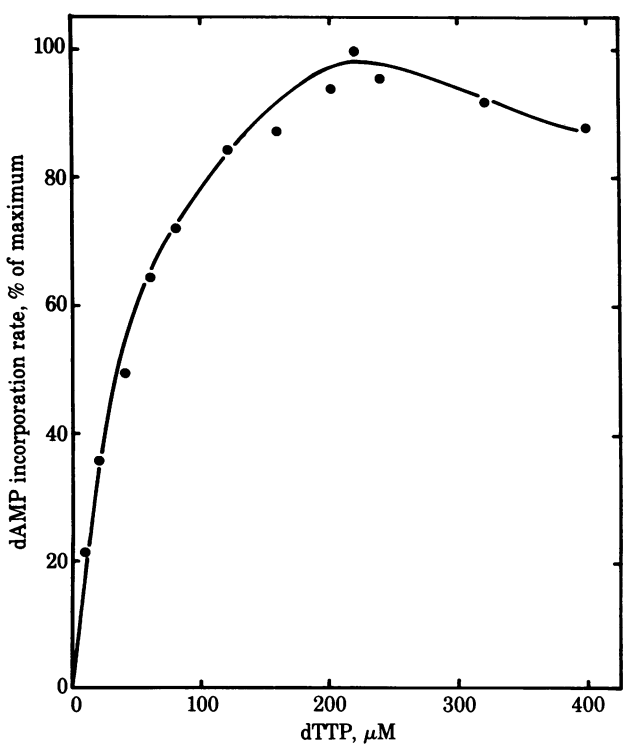

FIG. 1. Thymidine triphosphate concentration dependence of T4 DNA synthesis *in vitro*. Conditions were identical to those in the experiment of figure 3C of ref. 22, except that the concentrations of dATP, dGTP, and dCTP were held constant at 200 μM.

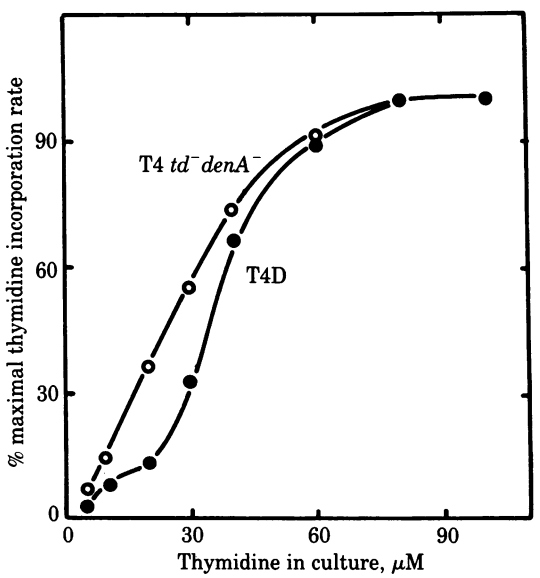

FIG. 2. Concentration dependence of thymidine incorporation into DNA. *E. coli* 201 was grown in glycerol/Casamino acids medium plus 100 μM thymidine at 37°C. At about 4×10^8 cells per ml, cells were centrifuged, washed, and resuspended at various thymidine concentrations. Cultures were infected with T4D or T4 *td8nd28*. At 3 min after infection [^{3}H]thymidine was added to 100 μCi/μmol (1 Ci = 3.7×10^{10} becquerels), and 50-μl samples of each culture were taken for determination of thymidine incorporation into DNA.

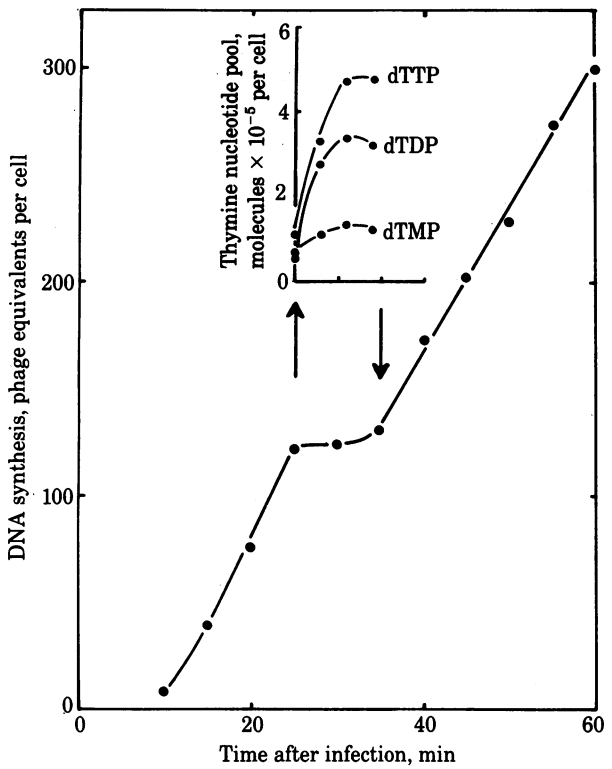

FIG. 3. Effect of a reversible blockage of DNA synthesis upon thymidine nucleotide pools and rate of synthesis. Cells were grown at 37°C in 100 μM thymidine, then centrifuged, washed, and transferred to fresh medium containing 100 μM thymidine. Infection was carried out at 30°C and at a multiplicity of seven with T4 *td8nd28tsL159*. At 3 min after infection [^{3}H]thymidine was added to 250 μCi/μmol, and 50-μl samples were taken at 5-min intervals for measurement of incorporation into DNA. At 25 min the culture was shifted to a 42°C water bath, and at 35 min it was returned to 30°C (indicated by arrows on the figure). At 0, 3, 6, and 9 min after shiftup, 2-ml samples were taken for determination of radioactivity in thymidine nucleotide pools.

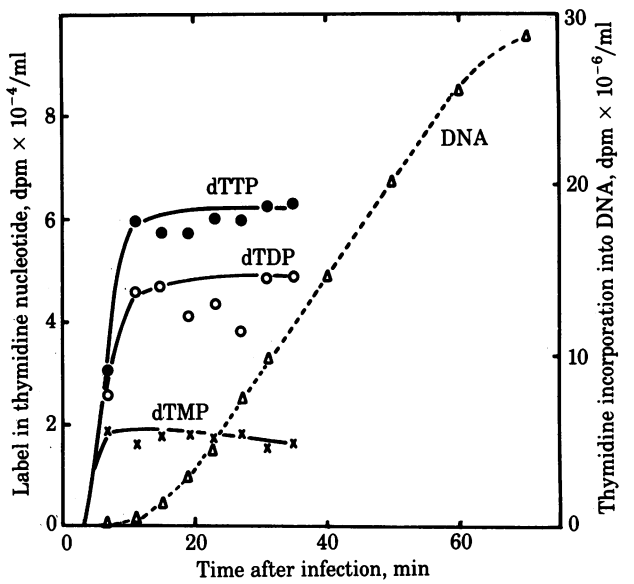

FIG. 4. Rate of labeling of thymidine nucleotide pools. Conditions were as described in the legend to Fig. 3, except for the phage strain used (T4 *td8nd28*), the temperature (37°C), and the times of sampling. Two-milliliter samples were removed as indicated for extraction and analysis of thymidine nucleotide pools, and 10-μl samples were removed for monitoring thymidine incorporation into DNA (broken line).

30°C in 100 μM thymidine accumulates thymidine nucleotides rapidly after a shift to 42°C, to nearly 5-fold higher than the preshiftup level. However, the rate of DNA synthesis after a return to 30°C is no higher than the preshiftup rate. Because all four dNTPs accumulate when gene *45* is blocked (24), the rate after shiftdown is probably not limited by availability of any precursor.

Results comparable to those of Fig. 3 were obtained in similar experiments using phage with temperature-sensitive mutations in genes *32* or *42* (data not shown).

The culture depicted in Fig. 3 displayed a DNA synthetic rate of 8.5 phage-equivalent units per min and a dTTP pool size at 30°C of 1.1×10^5 molecules per cell. Both values correspond closely to those observed in infections of wild-type bacteria by wild-type phage. Thus, the conclusion from Fig. 3, that the replication apparatus is saturated with dTTP in our system, is probably valid for wild-type infections as well.

Thymidine Concentration Dependence of dTTP Pool and DNA Synthetic Rate. We wished to carry out T4 *td8nd28* infections in the presence of various external thymidine concentrations and measure both the dTTP pool and the rate of DNA synthesis. At the minimal thymidine level that gave maximal rates of DNA synthesis we could, from pool measurements, determine the average intracellular dTTP concentration and, by comparison with the *in vitro* DNA synthesis data (Fig. 1), estimate the magnitude of the dTTP concentration gradient, if any, existing near replication sites. In order to do so, it was necessary to establish that radioactivity recovered in the extracted dTTP pool did indeed represent the pool size. Radioactive thymidine was added to a culture at 3 min after infection. At 4-min intervals thereafter samples were harvested and extracted, and radioactivity was measured in each thymine nucleotide pool. As shown in Fig. 4, it required about 8 min for the dTTP pool to reach its maximal radioactivity, and after that point little change was seen. Because exogenous deoxynucleoside represents the only source of thymine nucleotide pools in this experiment, we infer that the 8-min labeling interval suffices to completely turn over the preexisting unlabeled dTTP pool, and, hence, that samples taken for analysis after 11 min after infection accurately represent intracellular thymine nucleotide pools.

Fig. 5 shows the results of an experiment in which DNA synthetic rates and dTTP pool sizes were determined as a function of thymidine concentration in the same set of cultures (actually, the figure depicts averaged data from two identical experiments). The maximal rate of DNA synthesis in each experiment was achieved at 80 μM exogenous thymidine. The dTTP pool size at this concentration was 1.2×10^5 molecules per cell in one experiment and 1.13×10^5 molecules per cell in the other. If we use 1.17×10^5 as the average value, and if we use 1.8 μm^3 as the average volume of a T4-infected *E. coli* cell (27), then we can estimate that the minimal average intracellular dTTP concentration is about 65 μM in cells that are synthesizing phage DNA at the maximal rate.

DISCUSSION

Three significant findings emerge from this investigation: (*i*) Thymidine triphosphate concentrations at T4 DNA replication sites are in the range needed to saturate the replication apparatus; a minimal effective concentration of 200–240 μM is estimated from our experiments on DNA synthesis *in vitro*. (*ii*) This value is at least 3- to 4-fold higher than the estimated average intracellular dTTP concentration. (*iii*) Exogenous thymidine at high concentrations (80 μM or above) can completely bypass the two virus-coded endogenous routes to thymidine nucleotide formation and be incorporated at full specific activ-

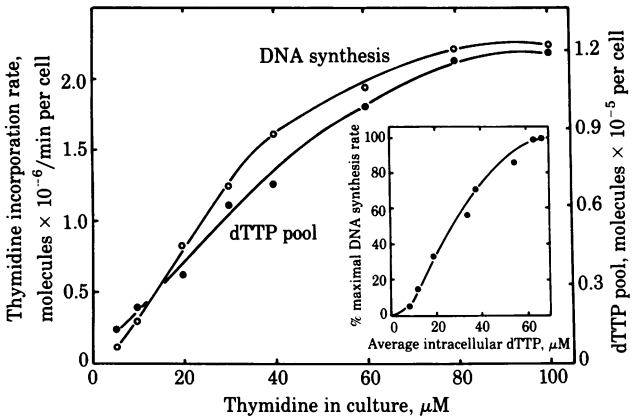

FIG. 5. Thymidine concentration dependence of dTTP pool and rate of DNA synthesis. Conditions were identical to those described for Fig. 4, except that the bacteria were centrifuged and washed before infection, and 2.5-ml cultures were infected with T4 *td8nd28* at each of the indicated thymidine concentrations. At 25 min, 2.0-ml aliquots were removed for dTTP pool size determinations (●), and at intervals 10-μl samples were removed for determination of rates of DNA synthesis (○). (*Inset*) DNA synthetic rate as a function of dTTP pool size (converted to average intracellular concentrations).

ity, even in infection by wild-type T4 phage.

From the data of Figs. 1 and 5 we tentatively conclude that a dTTP concentration gradient of at least 3- to 4-fold exists at intracellular DNA replication sites. The validity of this conclusion rests upon three assumptions: (*i*) that our estimates of average intracellular dTTP concentrations are reasonable, (*ii*) that rates of DNA synthesis in our thymidine-limited cultures are controlled by dTTP levels and not by the levels of other nucleotides, and (*iii*) that the purified protein system for *in vitro* DNA synthesis represents a suitable model for T4 DNA synthesis *in vivo*.

Assumption i. Because our dTTP pool size determinations are probably accurate (discussed in refs. 1 and 24), the main factors to consider here are the estimated volume of an *E. coli* cell and the fraction of that volume that is accessible to nucleotides. A value of 1 μm^3 is commonly taken as the volume of an uninfected *E. coli* cell in the midlogarithmic phase of growth (27, 28), although the precise volume varies with growth rate (28). About 80% of the volume of an *E. coli* cell is water, and presumably this represents a minimal fraction of the total cell volume that can be occupied by nucleotides. In a careful study, Freedman and Krisch (29) showed that *E. coli* cells more than double in volume after T4 phage infection, with most, but not all, of the increase coming from imbibition of water. Thus, most or all of the increased volume represents a compartment that should be accessible to nucleotides. Bacteria grown and infected with T4 under conditions similar to ours ultimately reached a mean cell volume of 2.2 μm^3 in their experiments. The value of 1.8 μm^3 used in our study represents the volume attained after 30 min of infection, which corresponds to our time of sampling for nucleotide pools. From the above considerations it seems that at least 85–90% of this volume should be accessible to nucleotides; if the remainder is actually inaccessible, then our average intracellular concentrations would be slightly underestimated.

Assumption ii. Deoxyribonucleotide biosynthesis is subject to a complex regulatory network involving enzyme activation and inhibition by dNTPs. Is it possible that the reduced pools of dTTP brought about by thymidine limitation lead to underproduction of other nucleotides, so that some property other than dTTP levels becomes rate-limiting? Although we have not measured the levels of other dNTPs in this study, we think that this possibility is remote. Consider, for example, the following scenario: dTTP is the prime positive effector for reduction of GDP by T4 ribonucleotide reductase (30). In principle, low dTTP levels could lead to dGTP pool shrinkage, through diminished reductase activity on GDP. However, the lowest dTTP pools we measured in the experiment of Fig. 5 correspond to values that still give near-maximal stimulation of GDP reductase activity *in vitro* (30). Other possible scenarios are even more unlikely. Thus, we feel that DNA synthesis is limited primarily by dTTP availability under our conditions of thymidine limitation.

Assumption iii. This is the hardest assumption to deal with in a completely convincing way. Our choice of the purified protein system as an *in vitro* model for T4 DNA replication was based upon our ability to control nucleotide levels precisely in this system, along with the fact that the seven-protein system closely approximates *in vivo* replication with respect both to rate and accuracy of DNA synthesis. Of course the milieu at DNA growing points *in vitro* must vary significantly from that *in vivo* with respect to ionic strength, viscosity, and hydrophobicity, and these could alter apparent affinities for dNTPs. However, two observations are significant: First, we have varied several conditions under which the *in vitro* assays are conducted, without effect on the requirement for at least 200 μM dTTP to saturate the system. Second, we take note of our earlier studies with sucrose-plasmolyzed T4-infected cells, a system that more closely approximates the intracellular milieu (2). This system requires dNTP concentrations of at least 400 μM each to achieve saturation. Because sucrose-plasmolyzed cells retain aspects of dNTP compartmentation characteristic of intact cells, we feel that the purified protein system is a more appropriate model. However, these considerations suggest that our estimate of a 3- to 4-fold concentration gradient may actually be overly conservative.

The biological significance of dNTP concentration gradients cannot be readily assessed. We believe that such gradients are generated, at least in T4-infected bacteria, by the action of a multienzyme complex that synthesizes dNTPs from more distal DNA precursors (6–10). This complex, as studied *in vitro*, behaves as though capable of maintaining as much as a 50-fold gradient of deoxyribonucleotide intermediates (7). The *in vivo* gradient which we have estimated from this study is considerably lower but, as stated earlier, this represents a minimal estimate. It is particularly interesting that the K_m for DNA polymerase as part of the seven-protein system is considerably higher than corresponding values for isolated T4 DNA polymerase. The half-saturating dTTP concentration for the seven-protein system is about 40 μM (Fig. 1), whereas purified DNA polymerase acting on salmon sperm DNA displayed K_m values in the 1–2 μM range for each of the four dNTPs (31). It is now well established from the work of Alberts and colleagues (32) that interactions between T4 DNA polymerase and other replication proteins change several important properties of polymerase—processivity, V_{max}, etc. Does the change in substrate affinity have any adaptive value to T4? Although it may be difficult to answer this question definitively until comparable data are available for other organisms, it is intriguing to consider the relationship between dNTP concentrations and the accuracy of replication.

Most published work relating dNTP concentrations to replication fidelity involves the generation of pool biases, either *in vivo* or *in vitro*; these presumably act by changing the ratios of nucleotides that are competing for incorporation at a given site. What considerations hold sway at a normal replication fork, where there are probably not large pool biases, but where the levels of all four dNTPs may be much higher than generally realized?

As pointed out largely by Fersht (14, 18) and by Clayton *et al.* (13), fidelity is controlled by several factors, including misinsertion frequency, probability of exonucleolytic removal of a mispaired nucleotide, and rate of addition of the next nucleotide. Fersht (14) has pointed out that under some circumstances the error rate can vary with increasing dNTP concentrations even if the ratios of competing nucleotides do not change. This can occur, for example, if the competing nucleotide and the next nucleotide to be added are the same, so that the error frequency shows second-order dependence on this nucleotide, but only first-order dependence on the correct nucleotide. In other words, misincorporation frequency would depend both upon competition for insertion at a given site and the frequency with which the next nucleotide is added, converting a misinserted nucleotide (which otherwise would be cleaved out by exonucleolytic turnover) to a misincorporated nucleotide. These and other factors (such as nucleotide sequence at the normal and mutant sites) make it difficult to predict the effects on fidelity of a generalized increase in dNTP levels. However, these factors indicate that nucleotide compartmentation must be taken into account in any treatment that would quantitatively relate mutation frequencies to intracellular dNTP concentrations as estimated from pool measurements.

A by-product of this study that should be useful to phage workers is our finding that exogenous thymidine is preferentially incorporated into DNA at high concentrations. This means that, with appropriate attention to experimental conditions, thymidine incorporation can be used in phage-infected bacteria as a valid quantitative indicator of DNA synthesis. We have not yet demonstrated preferential thymidine incorporation under conditions in which endogenous pools are expanded, for example, by a reversible blockade of DNA synthesis (1). However, it would be a simple matter to test this. Of greatest interest to us is the fact that our results portray a functional association between T4 thymidine kinase and other enzymes of DNA precursor biosynthesis. Why this should be evolutionarily advantageous to phage is not clear, because infected cells probably do not normally encounter high levels of thymidine, and endogenous pathways seem to operate at more than adequate rates. Nevertheless, our data are consistent with our as yet unpublished findings regarding physical association between thymidine kinase and other enzymes of deoxyribonucleotide metabolism.

Knowing the true effective concentrations of dNTPs in different organisms represents an important experimental objective because, as stated earlier, these factors undoubtedly contribute toward determining spontaneous mutation frequencies. However, it is an extraordinarily difficult objective to achieve, and this study represents but a small first step.

C.K.M. thanks Mr. G. W. Lasser for technical assistance and Drs. T. W. North and J. R. Allen for useful discussions and constructive criticism. This study was supported by National Institutes of Health Research Grants AI-15145 (to C.K.M.) and GM-24391 and funds from the Busch Foundation (to N.K.S.).

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