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Quantitation of cellular deoxynucleoside triphosphates

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ABSTRACT

Eukaryotic cells contain a delicate balance of minute amounts of the four deoxyribonucleoside triphosphates (dNTPs), sufficient only for a few minutes of DNA replication. Both a deficiency and a surplus of a single dNTP may result in increased mutation rates, faulty DNA repair or mitochondrial DNA depletion. dNTPs are usually quantified by an enzymatic assay in which incorporation of radioactive dATP (or radioactive dTTP in the assay for dATP) into specific synthetic oligonucleotides by a DNA polymerase is proportional to the concentration of the unknown dNTP. We find that the commonly used Klenow DNA polymerase may substitute the corresponding ribonucleotide for the unknown dNTP leading in some instances to a large overestimation of dNTPs. We now describe assay conditions for each dNTP that avoid ribonucleotide incorporation. For the dTTP and dATP assays it suffices to minimize the concentrations of the Klenow enzyme and of labeled dATP (or dTTP); for dCTP and dGTP we had to replace the Klenow enzyme with either the Taq DNA polymerase or Thermo Sequenase. We suggest that in some earlier reports ribonucleotide incorporation may have caused too high values for dGTP and dCTP.

INTRODUCTION

DNA replication and repair require each of the four canonical deoxyribonucleoside triphosphates (dNTPs). The relative amount of each dNTP is important for correct DNA synthesis (1–4). Genetic diseases result from mutations in anabolic enzymes (5,6) leading to deficiency of one or the other dNTP, and also from mutations in catabolic enzymes (2,7,8) resulting in the excess of one or several dNTPs. In recent years interest in the determination of dNTPs in cell extracts has increased greatly, stimulated by discoveries that have shifted the focus from the needs of nuclear DNA replication during S phase to the requirements of mitochondrial DNA maintenance in differentiated cells (9). In addition, the discovery that ribonucleotide reduction takes place also outside S phase (10,11) is inducible by DNA damage (12) and is a limiting source of dNTPs for mitochondrial DNA synthesis in quiescent cells (13) has highlighted the importance of measuring dNTPs in non-proliferating cells or in mitochondria. The sizes of the dNTP pools shrink about 10-fold as cells stop cycling and mitochondrial pools, which correspond to less than 10% of the overall cellular pools (14), reach levels below 1 pmol per million cells. Thus a sensitive and reliable method for the determination of minute amounts of dNTPs in cell extracts becomes indispensable to understand the mechanisms of nuclear and mitochondrial DNA replication and repair (15), mutagenesis (16) and apoptosis (17) and detect changes in the dNTP pools in genetically manipulated cells (18) and animal models of human diseases (19,20).

dNTPs in extracts from cultured cells or animal organs are usually quantified by two independent methods: high-performance liquid chromatography (HPLC) (10,21,22) or an enzymatic assay with a DNA polymerase (23,24). Each method has its problems and ideally both should be used in parallel. Often the small amounts of dNTPs in the extracts prohibit the use of HPLC and the more sensitive enzymatic assay then becomes the method of choice. More recently, highly sensitive LC-MS/MS methods were introduced (25,26). Unfortunately they are unsuitable for measurements of dCTP and dGTP (25) or dGTP (26) and thus do not provide the required spectrum of all four dNTPs. An additional weakness is that the specific radioactivity of dNTPs in isotope experiments cannot be measured.

The enzymatic method is based on the incorporation of labeled dATP (or dTTP) into defined synthetic oligonucleotides by a DNA polymerase (usually the *Escherichia coli* Klenow fragment). The oligonucleotides used for each dNTP are constructed such that the incorporation of labeled dATP (or dTTP) is proportional to the amount of the dNTP to be determined (23). As an example, Figure 1 shows the structure of the oligonucleotide for the dCTP assay. Elongation of the primer strand requires dCTP with the incorporation of

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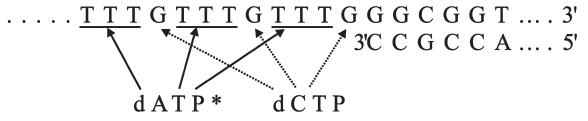

Figure 1. Enzymatic assay for dCTP. During incubation with a specific oligonucleotide template a DNA polymerase incorporates three molecules of labeled dATP for each dCTP converting them into an acid-insoluble form. The amount of dCTP is calculated from the incorporated radioactivitiy in a standard curve with known amounts of dCTP.

one molecule of dCTP promoting incorporation of three labeled dATPs. From the radioactivity incorporated into the oligomer and a standard curve with known amounts of non-radioactive dCTP one can then calculate the concentration of dCTP in the sample to be analyzed. In model experiments as little as 0.1 pmol of dCTP can be determined with great accuracy. The validity of the assay depends on the specificity of the polymerase, i.e. only dCTP should promote the incorporation of radioactive dATP. When dNTPs are analyzed in cell extracts, however, the specificity of the assay may be compromised by interfering nucleotides. Specificity rests not only on the selection of the correct dNTPs through base pairing with the template strand, but also on the ability of the polymerase to distinguish between nucleotides containing sugars other than deoxyribose. In particular ribonucleoside triphosphates (rNTPs) provide a complication in this respect.

We observed a lack of proportionality in the incorporation of labeled dATP in assays with different aliquots of extracts from quiescent cultured fibroblasts containing very small dNTP pools. When we attempted to increase the sensitivity of the enzymatic assay by increasing the concentration of the polymerase we obtained unrealistically high dNTP values (our unpublished data). At this point we remembered that already in the 1960s it was reported that the *E. coli* DNA polymerase could substitute rNTPs for dNTPs (27), with CTP and GTP being particularly effective (28). Incorporation was pronounced when Mn^{2+} replaced Mg^{2+} in the reaction. Subsequently it was shown (29) that the Klenow fragment of the polymerase used in our assays has a limited specificity for deoxyribose also in the presence of Mg^{2+}. We set out to study this problem in detail to find dNTP assay conditions without interference by ribonucleotides. Here we describe the results of these studies. We found that both dATP and dTTP could be determined with the Klenow fragment, provided care was taken to limit the concentration of the enzyme. For dCTP and dGTP also limiting concentrations of the Klenow enzyme led to erroneously high values. These two nucleotides could instead be assayed by substituting the Klenow enzyme by either Taq DNA polymerase or Thermo Sequenase.

MATERIALS AND METHODS

Enzymes

The Klenow fragment of the *E. coli* DNA polymerase I used in the assays described in 'Results' section was from Invitrogen (Life Science, San Giuliano Milanese, Italy). Other preparations of the Klenow enzyme were from Biolabs (Celbio, Pero, Italy) or Promega (Milano, Italy). The *E. coli* DNA polymerase I was from Promega. Taq DNA polymerase and Thermo Sequenase DNA Polymerase with *Thermoplasma acidophilum* inorganic pyrophosphatase was from GE Healthcare (Little Chalfont, UK).

Enzymatic assays, general conditions

The general conditions for the assays were those described by Sherman and Fyfe (23), including the preparation of the specific oligonucleotides used for each of the four dNTPs. We used, however, a 10-fold lower concentration of labeled dATP or dTTP. Briefly, the reaction mixture contained, in a volume of 0.1 ml, 0.1–4 pmol of the dNTP to be determined together with 40 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.25 μM oligonucleotide, 1.5 μg RNase A, 0.25 μM labeled dATP, 500–1000 cpm/pmol (or labeled dTTP for the dATP assay) and DNA polymerase. RNase A was included in the assay to remove any labeled RNA formed in metabolic experiments with ^3H- labeled nucleosides. The nature and the amount of the DNA polymerase used in the assay are given below for each separate dNTP determination.

After 60 min incubation 0.085 ml of the mix was spotted on circular disks of Whatman DE81 paper. After drying, the filters were washed three times for 10 min in large volumes of 5% Na_2HPO_4, once in distilled water and once in absolute ethanol. The retained radioactivity was determined by scintillation counting.

Enzyme concentrations for the different dNTPs

We used limiting amounts of the Klenow enzyme for dTTP and dATP assays and the Taq DNA polymerase for dCTP and dGTP assays. The chosen enzyme concentration must be balanced by the requirement of a close to linear dose–response curve in the range of the dNTP concentrations to be analyzed. It is advisable to test each new batch of enzyme to find the smallest amount of enzyme that for a given dNTP suffices to give a close to linear standard curve between 0.1 and 4 pmol of the dNTP. We found this range to be optimal for the analysis of dNTPs present in cell extracts. For our Klenow enzyme 0.2 units sufficed for the dTTP assay and 0.025 units for the dATP assay, with 60 min incubation at 37°C. For Taq DNA polymerase 2 units were required for both dCTP and dGTP, with 60 min incubation at 48°C. Once standardized, we used each batch of enzyme for months without change of conditions and without interference by rNTPs.

Cell extracts for dNTP assays

For the experiment described in Table 1 we prepared extracts from cycling and confluent normal human skin fibroblasts, grown in DMEM with Earle salts and glutamine and 10% fetal bovine serum (Invitrogen). Cultures were started at 0.3 million cells/10 cm dish with fresh medium being supplied twice weekly. Cycling cells were harvested after 48 h growth (14% S-phase,

determined by fluorescence-activated flow cytometry), confluent cells were harvested after 14 days in culture (1.8% S-phase). Dishes were cooled on ice, the cells were carefully washed free of medium and extracted with 2 ml/dish of ice-cold 60% methanol (8). After immersion for 3 min in a boiling water bath the methanolic extract was centrifuged and brought to dryness by centrifugal evaporation. The dry residue was dissolved in 0.2 ml of water and used for assays. We analyzed routinely two aliquots of different size per each sample to ascertain linearity of the assay.

HPLC chromatography

The eight canonical rNTPs and dNTPs were separated on a WAX weak anion exchange column (PolyLC, DTO Servizi, Spinea, Italy) by isocratic elution with 0.32 M KH_2PO_4, pH 5.0, containing 3% acetonitrile (flow rate 0.5 ml/min) (10). We found the following retention times (in min):dTTP, 15.8; UTP,18.8;dCTP, 25.1; CTP, 29.2; dATP, 35.4; ATP, 40.5; dGTP, 71.2 and GTP 83.4. Peaks in cell extracts could be identified from their positions and from their relative absorbances at 260 and 280 nm. When analyzed by HPLC the rNTPs used in the model experiments contained no detectable dNTPs.

RESULTS

CTP replaces dCTP for the Klenow polymerase

We substituted CTP for dCTP in an experiment with the oligonucleotide shown in Figure 1 to test if the ribonucleotide would promote the incorporation of labeled dATP. Figure 2 shows one of several typical experiments in which we measured the ability of 50 or 1000 pmol of CTP to support DNA replication in the absence of dCTP. The two amounts of CTP correspond approximately to those present in the aliquots of extracts from cycling or confluent cultured cells used for dCTP assays as described below in Table 1. In the ordinate of Figure 2 we have transformed the incorporated radioactivity into apparent pmol of dCTP calculated from a parallel standard curve with known amounts of dCTP. Incorporation of radioactivity increased not only with the amount of CTP, but also with the amount of the polymerase and to a somewhat lesser extent with that of labeled dATP. Thus, at the lower enzyme and dATP concentrations, 50 pmol CTP mimicked the presence of almost 1 pmol of dCTP whereas 1000 pmol mimicked 3 pmol. At the higher dATP and enzyme concentrations the apparent dCTP increased to 3 and 20 pmol, respectively. These data clearly show that with the Klenow polymerase the assay of dCTP in cell extracts might be seriously flawed by the presence of CTP. We obtained similar results when GTP or ATP was tested in place of CTP in the absence of the cognate deoxynucleotide (data not shown).

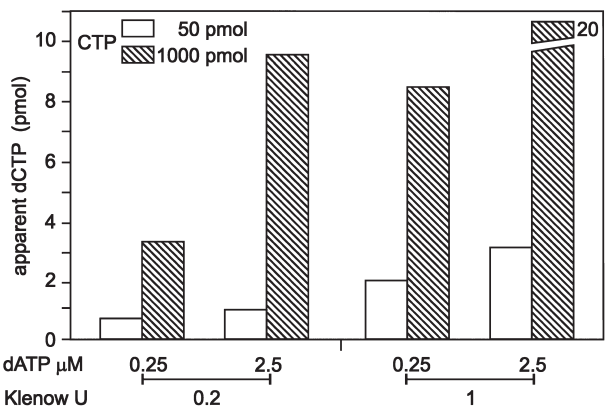

Figure 2. CTP replaces dCTP during assay with the Klenow DNA polymerase. In the absence of dCTP we incubated CTP (50 or 1000 pmol) with the Klenow enzyme (0.2 or 1 unit) together with labeled dATP (0.25 or 2.5 μM) as described in 'Materials and Methods' section and determined the incorporation of radioactivity into the oligonucleotide shown in Figure 1. The recovered acid-insoluble radioactivity was transformed into apparent pmol dCTP (ordinate) as calculated from a parallel standard curve with known amounts of dCTP.

rNTPs contribute to dNTP assays by the Klenow polymerase

We found that rNTPs in amounts present in cell extracts increased the incorporation of labeled dATP (dTTP in the case of ATP) into oligonucleotides during assays of dNTPs, leading to erroneously high values for the dNTPs. The magnitude of the effect depended on the concentrations of the rNTP, the enzyme, and the labeled dATP. Each rNTP/dNTP pair showed in principle this behavior, but with considerable individual quantitative differences. Figure 3 shows typical model experiments with known amounts of standard nucleotides for each rNTP/dNTP pair. In these and other nonpublished experiments the concentrations of CTP, GTP and UTP were two or three orders of magnitude in excess over the corresponding dNTPs and that of ATP four orders of magnitude. In all cases CTP (Figure 3A) and GTP (Figure 3B) at higher concentration gave the largest disturbances with an up to 10-fold increased value for dCTP and a 4-fold increase for dGTP, respectively. The data in Figure 3A and B were obtained with 0.2 units of enzyme. In additional experiments we tested different concentrations of polymerase and dNTPs, and again found interference by CTP and GTP (data not shown). The effects of UTP (Figure 3C) and ATP (Figure 3D) were smaller and minimal at the lowest enzyme concentration. Three different commercial preparations of the Klenow enzyme and one preparation of *E. coli* DNA polymerase I were investigated with similar results, albeit with minor quantitative differences (data not shown). From these results we conclude that the Klenow enzyme is not suitable for dCTP and dGTP assays but can be used for the assay of dTTP and dATP. With these two dNTPs, care must be taken to determine in preliminary model experiments a concentration of Klenow polymerase sufficiently low to avoid the interference by the rNTPs. One must of course also ensure that the enzyme concentration gives an appropriate standard curve in the desired dTTP and dATP concentration range. The

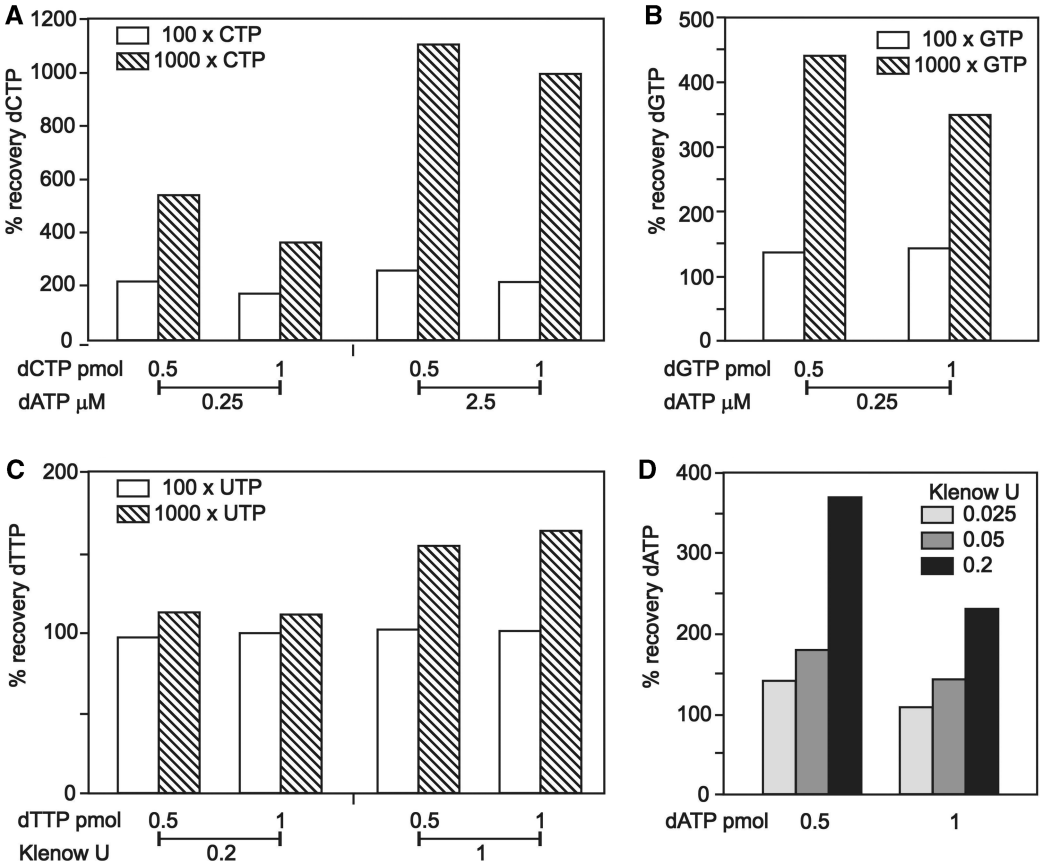

Figure 3. Effect of rNTPs on the recovery of dNTPs in assays with the Klenow polymerase. (**A**) CTP (at 100- or 1000-fold higher concentration than dCTP) in dCTP (0.5 or 1 pmol) assay with 0.2 units of enzyme and either 0.25 or 2.5 μM labeled dATP. The ordinate shows the increased apparent recovery of dCTP in the assay, due to misincorporation of CTP in the polymer. (**B**) GTP in dGTP assay. Conditions as in A. (**C**) UTP in dTTP assay. We tested two enzyme concentrations (0.2 and 1 unit) at 0.25 μM labeled dATP. Other conditions as in A. Very similar values for dTTP recovery were obtained also with 200- and 2000-fold excess UTP. (**D**) ATP in dATP assay. Quantitation of dATP (0.5 or 1 pmol) in the presence of 5000 pmol ATP and 0.25 μM labeled dTTP at various enzyme concentrations (0.025, 0.05 or 0.2 units).

original assay prescribes the use of 0.5 units of polymerase per 0.1 ml assay volume. We found this to be an excess. With our commercial Klenow enzyme we determined 0.025 units to be optimal for the dATP assay and 0.2 units for dTTP.

Taq DNA polymerase or Thermo Sequenase are suitable for dCTP and dGTP assays

The discrimination of the Klenow polymerase between the ribo- and deoxyribonucleotides of guanine and cytosine was not sufficient to permit the use of the enzyme to assay dCTP and dGTP. We therefore turned to other DNA polymerases and tested their suitability. We found that both Taq DNA polymerase and Thermo Sequenase showed the required discrimination. Figure 4A and B report representative dCTP and dGTP assays, respectively, with Taq DNA polymerase. In both cases the addition of the ribotides in 100- or 1000-fold excess over two concentrations of the corresponding dNTPs only marginally affected the result of the assays. Figure 4C shows a similar experiment with the Thermo Sequenase in a dGTP assay in the presence of a 200–1000-fold excess of GTP. These experiments were not run at the optimal temperature of the thermostable enzymes but at 48°C to avoid melting of the oligonucleotide primers.

Comparison of dNTP determinations in cell extracts by different assays

To establish the validity of the 'new' DNA polymerase assay we extracted dNTP pools from cycling and confluent human fibroblasts in culture and determined all four dNTP pools by the polymerase assay with the Klenow enzyme as described in the original communication (23) and by the new method described here (Table 1). In cycling cells we also determined rNTPs and dNTPs by a HPLC method that separates all eight ribo- and deoxyribonucleoside triphosphates (10). By the same procedure we measured rNTPs in extracts from confluent cells. Here the concentration of dNTPs was too low to be measured with accuracy by HPLC due to interference by unknown peaks and neighboring rNTPs. For dNTPs in cycling cells the agreement between HPLC and the new polymerase assay was excellent (Table 1). In contrast, the original method gave twice the amount of dCTP and dGTP, a small increase of dATP and no difference for dTTP. These relations agree with the

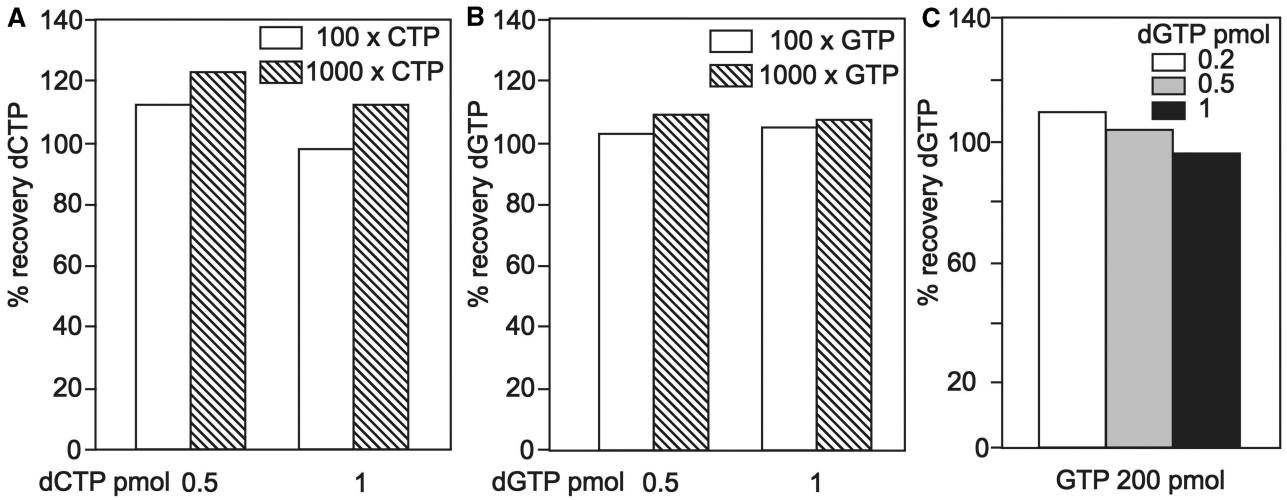

Figure 4. Assays of dCTP and dGTP with Taq DNA polymerase or Thermo Sequenase. (**A**) Effect of CTP (100- or 1000-fold excess) in dCTP (0.5 or 1 pmol) assay at 0.25 μM dATP with 2 units Taq DNA polymerase. (**B**) GTP in dGTP assay. Conditions as in A. (**C**) Thermo Sequenase in dGTP assay. dGTP (0.2–1 pmol) was assayed with 2 units Thermo Sequenase in the presence of 200 pmol GTP. General conditions as in Figure 2.

Table 1. Deoxyribonucleoside and ribonucleoside triphosphates in extracts from cycling and confluent human fibroblasts. dNTPs were determined by the original enzymatic method (23), by the enzymatic method described here or by HPLC. rNTPs were measured by HPLC. The numbers represent pmol of each nucleotide per million cells

	(d)ATP	(d)GTP	dTTP(UTP)	(d)CTP
Cycling cells, dNTPs				
Original assay	57	32	92	89
New assay	47	18	92	44
HPLC	45	18	103	44
Cycling cells, rNTPs	15 200	2600	4000	1650
rNTP/dNTP ratio	350	140	40	40
Confluent cells, dNTPs				
Original assay	17	6	8	18
New assay	8	1.2	8	6
Confluent cells, rNTPs	11 600	2400	2800	970
rNTP/dNTP ratio	1450	2000	350	160

relative increases induced by ribonucleotides in the model experiments of Figure 3. Turning now to the confluent cells we notice first that all four dNTP pools were about 10 times smaller than in cycling cells. The four ribonucleoside triphosphates in contrast decreased only less than half. The discrepancy between the 'old' and new assays is now larger. The values in the 'old' assay are more than 3-fold larger for dCTP and dGTP and also dATP is 2-fold increased whereas the amount of dTTP is the same. The larger differences between the two methods in confluent cells reflect these cells' higher rNTP/dNTP ratios that enhance the unspecific rNTP incorporation in the oligonucleotide by the Klenow polymerase.

DISCUSSION

The concentration of the four dNTPs in eukaryotic cells is very low and undergoes large variations with the cell cycle, as illustrated here by the results in Table 1. Comparisons of pool sizes from normal cells with those of cells whose nuclear or mitochondrial DNA synthesis is compromised by mutations, genetic manipulations or diseases involve both logistic and analytical problems. One very important logistic problem that unfortunately is not always considered is to choose cell populations in equal growth phases for a comparison.

In this article we address an important analytical problem arising from the lack of specificity of the DNA polymerase in the enzymatic assay for the determination of dNTPs. The polymerase generally used for the assays is the Klenow DNA polymerase, together with oligonucleotides specifically designed for the assay of each dNTP (23). We found in model experiments that this enzyme to a small extent can substitute rNTPs for dNTPs for incorporation into the oligonucleotide. The degree of misincorporation differs for the four ribonucleotides. For each rNTP the major factors determining the extent of misincorporation were (i) the inherent specificity of the polymerase for the dNTP (ii) the concentration of the enzyme in the assay and (iii) the concentration of the rNTP in the sample relative to that of the dNTP.

In our model experiments with known amounts of standard nucleotides the inherent specificity of the Klenow enzyme was high for dTTP and lowest for dCTP and dGTP, with dATP occupying an intermediate position. These data fully agree with the earliest results concerning a low specificity of the *E. coli* DNA polymerase for dCTP and dGTP in the presence of Mn^{2+} (27) and later similar results with various polymerases and Mg^{2+} (30,31). In these experiments the inherent specificity of the Taq DNA polymerase was almost 10 times higher than that of the Klenow enzyme (31).

By limiting the concentration of the enzyme during the reaction we could completely avoid misincorporation of UTP or ATP and use the Klenow enzyme for assays of dTTP and dATP. This expedient was not effective for dCTP and dGTP and in these assays we had to substitute the Klenow enzyme with either Taq DNA polymerase or Thermo Sequenase. These two polymerases have a higher specificity for dNTPs but their concentrations must again be limited as at higher concentrations they also showed a tendency to incorporate rNTPs (our unpublished data).

The third factor that influenced the degree of misincorporation is the rNTP/dNTP ratio in the cell extract. In confluent human fibroblasts this ratio is very large, ranging from 160 for CTP/dCTP to 2000 for GTP/dGTP (Table 1) and may favor extensive misincorporation during the assay of dGTP.

These three factors limit the usefulness of the original DNA polymerase assay that under adverse circumstances may erroneously give a biased composition of the four dNTP pools, with a correct value for dTTP but a large overrepresentation of dCTP and dGTP. In particular cells and organelles with small dNTP pools are prone to suffer from this bias (cf Table 1). We believe that this may explain exceedingly high dGTP/dTTP ratio in mitochondria from rat organs (32) obtained with the polymerase assay. Such a skewed ratio could not be confirmed later (24) by HPLC analysis of mouse liver mitochondria.

In conclusion the modifications introduced here into the enzymatic assay should make it possible to avoid a major obstacle in the determination of the four canonical dNTPs which may lead to an overestimation of pool sizes. In general the DNA polymerase assay for dNTPs is used to provide still images of pool sizes of individual dNTPs. We wish to emphasize that the method also permits, in combination with isotope-flow experiments, to follow dynamics of dNTP pools, such as pool turnover, exchange of pool components between mitochondria and cytosol and *in situ* reaction rates (18,33).

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Conflict of interest statement. None declared.

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