Deoxynucleotide-interconverting enzymes and the quantification of deoxynucleoside triphosphates in mammalian cells

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We have demonstrated that methanol extracts of human cells are heterogeneous with regard to content of dNDP (deoxynucleoside diphosphate) and dNMP (deoxynucleoside monophosphate) kinases. The presence of these enzymes can affect the reliability of techniques used to measure intracellular pools of deoxynucleotides. An optimized extraction procedure and enzymic assay for dNTP species in haematopoietic cells are described which provide sensitivity to measure 0.1–40 pmol of dATP, dTTP and dGTP, and 1.0–40 pmol of dCTP. The extraction and assay give linear results with $(2.5\text{--}15) \times 10^6$ nucleated cells and $(0.1\text{--}1.5) \times 10^9$ red blood cells. Under these conditions, extracts equivalent to $\sim 0.5 \times 10^6$ nucleated haematopoietic cells catalyse the phosphorylation of 0–8% of dNDP and dNMP standards to dNTP and incorporate them into deoxynucleotide polymer under circumstances where 100% of an equimolar dNTP standard would be incorporated. By contrast, extracts of 0.4×10^6 HeLa cells totally converted dADP, dTDP and dGDP into dNTP with subsequent polymerization. Conversion of dCDP was somewhat less efficient. The results demonstrate conclusively that the activities of deoxynucleotide interconverting enzymes differ in different types of human cells. They can interfere with assay of nucleotides, but may not do so in many types of cell extracts. In particular, dNTP concentrations can be measured in human haematopoietic cells after extraction with 60% (v/v) methanol and are not artificially elevated by deoxynucleotide interconversions. It is apparent that extraction and assay procedures for measurement of dNTP species should be analysed for each cell type in order to minimize contaminating enzyme activities and ensure accuracy of dNTP quantification.

Quantification of deoxynucleoside triphosphates (dNTP species) in human cells has become an important practical problem since the recent description of hereditary abnormalities of deoxynucleotide metabolism in man (Coleman *et al.*, 1978; Cohen *et al.*, 1978). Accurate measurements are essential to investigators developing models of the metabolic disorders *in vitro* and describing the biochemical lesions in patients. Deficiencies of adenosine deaminase and purine nucleoside phosphorylase activities which result in accumulation of dATP or dGTP have been described in humans.

Because of the discrepancies in the literature concerning measurement of dNTP species in cells,

we have critically evaluated extraction and assay procedures. Of particular relevance is the fairly recent report by North *et al.* (1980) that methanol and HClO_4 extracts of HeLa cells either degrade deoxynucleotides or contain large quantities of kinases and nucleases. The combination of kinases and nucleases degrades and re-utilizes the DNA template in dNTP assays, thereby producing artificially high dNTP levels. North *et al.* (1980) recommended a two-step extraction procedure to eliminate most of the problems, but tested it only with HeLa cells. We present here evidence that methanol extracts of normal peripheral blood cells, leukaemic cells and lymphoblast cell lines do not contain significant amounts of enzymes which affect measurement of dNTP pools. These cells differ markedly from HeLa cells. It may thus be inferred that different cell types pose different problems in

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deoxynucleotide assay and that several procedures must be tested to optimize quantification of dNTP species in a specific circumstance.

Existing methods for measurement of dNTP species in the picomole range are based on the enzymic synthesis of DNA as first described by Solter & Handschumacher (1969). In this assay, dNTP species are the limiting factors for DNA synthesis when a DNA-dependent DNA polymerase (*Escherichia coli* DNA polymerase I), Mg^{2+} and nuclease-treated calf thymus DNA as primer template are present. To measure a particular dNTP, the other three dNTP species, one of which is radioactive, are added in non-limiting amounts. The polymerization will proceed until essentially all of the limiting dNTP is incorporated. The amount of the limiting dNTP can be determined by measurement of the radioactivity in the product. Sensitivity of the dNTP assay can be markedly improved by the substitution of high-molecular-weight double-stranded co-polymers for native DNA (Skoog, 1970; Lindberg & Skoog, 1970). Further improvement in sensitivity can be achieved by substitution of *Micrococcus luteus* DNA polymerase for *E. coli* polymerase (Tattersall & Harrap, 1973; Walters *et al.*, 1973). The variety of assay conditions employed has resulted in widely divergent reports of amounts of intracellular deoxynucleotides. For example, the concentrations of dATP and dTTP in resting human lymphocytes from peripheral blood have been reported to range from 0.2 and 0.01 (Munch-Petersen *et al.*, 1973) to 2.0 and 2.5 (Tattersall *et al.*, 1975) pmol/ 10^6 cells respectively. Likewise, to reports (Lowe & Grindey, 1976; Kinahan *et al.*, 1979) offer concentrations (pmol/ 10^6 cells) in the murine leukaemia cell line L1210 of 36 and 6.5 for dATP, 86 and 55 for dTTP, 36 and 4.4 for dGTP, and 56 and 30 for dCTP. We report some of the effects of various assay conditions on quantification of dNTP species. We recommend standard conditions that take into account the commercial availability of reagents and the level of sensitivity desired in the assay.

Experimental procedures

Materials

Highly purified *E. coli* DNA polymerase I [4409 units (one unit is the enzyme activity which produces in 30 min under assay conditions the incorporation of 10 nmol of total nucleotide into an acid-precipitable fraction)/mg of protein] and *M. luteus* DNA polymerase (325 units/mg of protein) were purchased from Boehringer-Mannheim Biochemicals, Indianapolis, IN, U.S.A., and Miles Laboratories, Elkhart, IN, U.S.A., respectively. 3H -labelled deoxynucleoside triphosphates were from New England Nuclear Corporation, Boston,

MA, U.S.A. Unlabelled oligodeoxynucleotides were obtained from P-L Biochemicals, Milwaukee, WI, U.S.A. Deoxyribonuclease-treated DNA was prepared as described by Aposhian & Kornberg (1962). Several different lots of poly[d(A-T)], poly[d(I-C)] and poly[d(G-C)] were purchased from P-L Biochemicals and Miles Laboratories. Polymers were dissolved in phosphate-buffered saline (0.1 M-NaCl/0.02 M-potassium phosphate, pH 7.0) to ensure the presence of the double-stranded configuration.

Cellular materials

Leucocytes were separated from erythrocytes by dextran sedimentation of blood treated with heparin or EDTA to prevent coagulation. Granulocytes were separated from mononuclear cells (lymphocytes and monocytes) by centrifugation of leucocytes on Ficoll/Hypaque gradients (Coleman *et al.*, 1978). Purified cells were washed twice with phosphate-buffered saline, cell concentration determined, and frozen as pellets at -70°C . An established line of human leukaemic cells, K562, was obtained from Dr. C. Lozzio, University of Tennessee, Knoxville, TN, U.S.A., and grown in RPMI 1640 tissue-culture medium containing 10% (v/v) foetal-calf serum. Lymphoblastoid cell line GM2294 was obtained from the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ, U.S.A. HeLa S3 cells were grown in Dulbecco's medium supplemented with 10% foetal-calf serum on 75 cm² plates.

Extraction of dNTP species

For extraction of deoxynucleotides, 50 μl of 10 mM-sodium tripolyphosphate was layered over a frozen pellet of 2.5×10^6 – 1.5×10^7 nucleated cells or 1×10^8 – 1.5×10^9 erythrocytes. Sodium tripolyphosphate improved the stability of dNTP and enhanced reproducibility of the yield. Methanol to a final concentration of 60% and a final volume of 2 ml was then added and the samples were left at 30°C for 15 min with occasional mixing (Skoog, 1970). The suspensions were then kept at -20°C overnight, followed by centrifugation at 8000 g for 2 min. The supernatant fractions were removed and dried at 30°C using a Buchler vortex evaporator. The residue was dissolved in 250–300 μl of water and clarified by centrifugation at 8000 g. Supernatant fractions were stored at -70°C until assay. HClO_4 extractions were by the method of North *et al.* (1980).

Enzymic assay of dNTP species

The assays for dNTP species are major modifications of techniques described by Solter & Handschumacher (1969), Skoog (1970) and Lindberg & Skoog (1970). The reaction mixture contained (final concentrations): 50 mM-Tris/HCl,

pH 7.8 at 37°C, 5 mM-MgCl₂, 1 mM- β -mercaptoethanol, 50 μ g of bovine serum albumin/ml, 0.5 A_{260} unit (20 μ g/ml) of primer-template/ml, 1.5 units of DNA polymerase/ml, and 10 μ M of the dNTP species in excess. The final reaction volume was 125 μ l. For assay of dATP and dTTP, poly[d(A-T)] was used as primer-template and either [³H]dTTP or [³H]dATP, as appropriate, was used as the dNTP in excess. For assay of dGTP, poly[d(I-C)] was the primer-template and [³H]dCTP was the labelled dNTP. When poly[d(I-C)] was used for assay of dCTP, [³H]dGTP was the dNTP in excess. Use of poly[d(G-C)] as template proved unsatisfactory, owing to the non-linearity of curves relating incorporation to quantity of dNTP added. [³H]dNTP species were used at specific radioactivities between 150 and 400 c.p.m./pmol. Incubations were carried out at 37°C for 60 min. Reactions were terminated by spotting portions in duplicate on Whatman GF/C squares and dropping them into cold 5% (w/v) trichloroacetic acid with 1% (w/v) sodium pyrophosphate. The squares were processed for liquid-scintillation counting of radioactivity as previously described (Chang & Bollum, 1971). Under these conditions dATP, dGTP and dTTP can be assayed in a linear range from 0.1 to 40 pmol if *M. luteus* polymerase is employed. dCTP can be assayed over the range 1–40 pmol.

High-pressure liquid chromatography

Analyses of deoxynucleotides were carried out by using a Varian 5000 high-pressure liquid chromatograph. A gradient elution was performed with a Whatman Partisil-SAX anion-exchange column (2 mm $\times$ 25 cm) at 22°C. The buffers for the gradient were: (A) 7 mM-potassium phosphate/7 mM-KCl, pH 4.0, and (B) 0.25 M-potassium phosphate/0.50 M-KCl, pH 5.0. The flow rate was 120 ml/h and the gradient program was: 100% buffer A for 5 min; linear increase to 100% buffer B during 35 min; 100% buffer B for 10 min. The column was then recycled. For determination of radioactivity in

material from the column, fractions containing 0.5 ml were added to a water-soluble scintillation fluid (3a70B; Research Products International Elk Grove Village, IL, U.S.A.) and counted for radioactivity.

Results

Kinetic parameters

There is wide variation in the concentration of non-rate-limiting dNTP species recommended by various authors for use in the enzymic assay of dNTP species, as well as in the concentration and type of template. We measured the kinetic parameters of the enzymic assay in order to estimate optimal reaction conditions (Table 1). DNA polymerase from *E. coli* was used, and assay conditions are described in the Experimental procedures section. DNA polymerase from *M. luteus* is kinetically similar to DNA polymerase from *E. coli* (Tattersall & Harrap, 1973). The apparent K_m of activated DNA as primer-template is 2 μ g/ml or approx. 0.5 A_{260} unit/ml. For synthetic polymers, the K_m of poly[d(A-T)] is 0.3 μ g/ml and for poly[d(I-C)] the K_m is 1 μ g/ml. The apparent K_m values for dNTP species range from 0.3 to 0.7 μ M. The concentrations used in our assays, 10 μ M for dNTP species and 20 μ g/ml for primer-templates respectively, are at least 10 times the K_m values.

Primer-template

The time course of incorporation of dNTP into primer-template has been examined extensively. Under the conditions of our standard assay, the polymerization reaction generally reached a maximal extent of incorporation after 20 min of incubation. There was no loss of incorporated [³H]-deoxynucleotide during incubation for as long as 120 min. The time of incubation for the standard assay is 60 min. It must be noted that some lots of poly[d(I-C)] do not give linear results in the dCTP assay, apparently owing to very slow polymerization.

Table 1. Kinetic constants for the DNA polymerase-mediated assay of deoxynucleotides

Substrate ...	activated DNA		Poly[d(I-C)]		Poly[d(A-T)]	
	K_m (μ M)	V_{max} *	K_m (μ M)	V_{max}	K_m (μ M)	V_{max}
dNTP						
dATP	0.64	730	—	—	0.39	670
dGTP	0.40	840	0.33	1500	—	—
dTTP	0.65	1040	—	—	0.36	1100
dCTP	0.37	1010	~3.7†	~490	—	—

* Units are nmol of dNTP incorporated/30 min per unit of polymerase.

† When poly[d(I-C)] is substrate, the polymerase has low affinity for dCTP. The K_m and V_{max} cannot be determined accurately because the double-reciprocal plot used to analyse Michaelis-Menten kinetics does not give a straight line.

The choice between activated DNA and synthetic polymer as primer-template for the assay of dNTP species is largely a matter of convenience and level of sensitivity desired. Although both templates give linear results, background incorporation is 2–4-fold higher with activated DNA than with synthetic polymer. Correspondingly, the ratio of net radioactivity counts above background to background is severalfold higher for polymer than for activated DNA for a given amount of standard dNTP, except for poly[d(I-C)] when dCTP is assayed. Since the sensitivity and reproducibility of radiometric assays are improved as the ratio of net counts to background increases, synthetic polymer is the preferred primer except when assaying dCTP. No synthetic polymer has proved uniformly satisfactory for assay of dCTP.

Extraction and assay of deoxynucleotides

Standard curves relating the amount of limiting dNTP to the net c.p.m. incorporated are shown in Fig. 1. The assay shown is linear with the four deoxynucleotides, dATP, dGTP, dCTP and dTTP, over the range 1–40 pmol. The lower limit of sensitivity is approx. 0.1 pmol for dATP, dTTP, and dGTP and 1.0 pmol for dCTP (due to high background). The data of Fig. 2 show the relationship between the amount of dATP detected (as c.p.m. incorporated) and the number of cells extracted in 2 ml of 60% methanol or the amount of extract in the assay. In Fig. 2(a) this relationship is linear over the range $(2.5\text{--}15) \times 10^6$ K562 cells; likewise, in Fig. 2(b), a linear relationship exists over the range

$(0.1\text{--}1.5) \times 10^9$ red cells. The data of Fig. 2 include many overlapping points from different numbers of cells extracted. The high degree of correlation observed ($r = 0.994$ and 0.984 for Figs. 2a and 2b respectively) indicates that both the numbers of cells extracted and the amount of extract in the assay are linear over the range described above. Above these numbers of cells the efficiency of extraction of deoxynucleotides from cells decreases, although the assay is still linear. Similar results are obtained for the other three dNTP species and with lymphocytes and lymphoid-cell lines. Measurements of dNTP species in normal peripheral blood lymphocytes and red blood cells are quite consistent from assay to assay. The mean ($\pm$ S.D.) dNTP pool sizes in adult

Fig. 1. Standard curves for assay of the four deoxy-nucleotides

Points represent the means of duplicate determinations. Duplicates agreed to within $\pm 5\%$ of the mean. (a) Covers the range 0–10 pmol of dNTP, whereas (b) covers the range of 10–100 pmol of dNTP. ●, dATP; ▲, dGTP; ■, dCTP; ○, dTTP.

Fig. 2. Relationship between amount of cell extract in the assay and the amount of dATP detected

The amount of extract is expressed in terms of the number of cells to which it corresponds. (a) 2.5, 5.0, 7.5, 10, 12.5 and 15, all $\times 10^6$, K562 cells were extracted in 2 ml of 60% methanol. Four different amounts of each extract were assayed. Each symbol represents one of these extracts. (b) 0.1, 0.3, 0.5, 1.0 and 1.5, all $\times 10^9$, red blood cells were extracted in 2 ml of 60% methanol. The assay procedure was as for (a).

lymphocytes are (pmol/ 10^6 cells): dATP, 4.8 (± 2.0 , $n = 7$); dGTP, 6.1 (± 3.3 , $n = 7$); dTTP, 1.4 (± 0.4 , $n = 6$); dCTP, 4.2 (± 3.4 , $n = 4$) (Hutton *et al.*, 1981). Pool sizes of the four dNTP species in red blood cells range between 0.1 and 0.2 pmol/ 10^6 cells. All measurements of dNTP species in cell extracts require assay of at least three different concentrations of extract to establish that the relationship between amount of extract and incorporation of dNTP is in the linear range. The quantity of dNTP in the extract is calculated from the slope of the resulting line.

Enzymic assay of dNTP species in normal human peripheral blood cells, leukaemic cells, and cell lines

Methanol extracts of Hela cells contain nucleoside diphosphokinase, deoxyribonucleoside monophosphokinases and nuclease (North *et al.*, 1980). We have found that extracts of peripheral blood lymphocytes and lymphoblast-cell lines may also contain low levels of these types of enzymic activities. In Figs. 3(a) and 3(b) are shown standard curves for the assay of dATP and dADP in the presence or absence of extracts from normal human lymphocytes and a lymphoblast cell line. In the absence of kinases the dADP should not be detected, because DNA-dependent DNA polymerases do not utilize deoxynucleoside diphosphates. The dATP standard curves are linear, at least over the 1–40 pmol range. When cell extract is added to the standard dATP, the effect is additive and the slope of the curve is not changed (Fig. 3). The slight amount of incorporation of dADP into DNA polymer in the absence of cell extract (~7% of the equimolar dATP standard) indicates the presence of nucleoside diphosphokinase in our *M. luteus* DNA polymerase. Both Figs. 3(a) and 3(b) show an increased incorporation of radiolabelled deoxynucleotide into polymer with increases in the amount of dADP added. The maximum amount of extract added to the dADP standards was equivalent to 0.53×10^6 cells (Fig. 3a) and 1.8×10^6 cells (Fig. 3b). These caused an additional 7.6% and 3.5% incorporation of radiolabel respectively into polymer. The increased incorporation of dADP into polymer in the presence as compared with the absence of cell extract indicated the presence of kinases in the cell extract.

Fig. 4 presents a similar series of experiments measuring the effect of extracts of the leukaemic cell line K562 on incorporation of the four dNDP species into polymer. Addition of dNDP affects each of the four assays for dNTP similarly. The nucleoside diphosphokinase activity in the preparation of *M. luteus* DNA polymerase added to the assays converts the dNDP species into the corresponding dNTP species. The dNTP species are then polymerized. Relative stimulation of the dNTP assays by

Fig. 3. Effect of methanol extracts of lymphocytes and lymphoblasts on incorporation of exogenous dATP and dADP into poly[d(A-T)].

Several different preparations of extracts were tested and are indicated by different symbols: dATP (○), dADP (●), dATP + extract (▼), dADP + extract (△, ▲, ▽). (a) Extracts of peripheral blood lymphocytes (△, 0.13×10^6 cells; ▲, 0.27×10^6 cells; ▽, 0.53×10^6 cells). (b) Extracts of GM2294 lymphoblasts (△, 0.45×10^6 cells; ▼, 1.8×10^6 cells).

Fig. 4. Effect of methanol extracts of K562 cells on incorporation of dNDP to polymers of deoxynucleotides

○, dNTP; ●, dNDP; △, dNDP + extract of 5×10^5 K562 cells. (a) Deoxynucleotides of T, poly[d(A-T)]; (b) deoxynucleotides of C, poly[d(I-C)]; (c) deoxynucleotides of A, poly[d(A-T)]; (d) deoxynucleotides of G, poly[d(I-C)].

dNDP species are: 3.5% for dADP, 4.0% for dGDP, 10% for dTDP, and 5.3% for dCDP. These are each calculated as percentage incorporations compared with an equimolar amount of the corresponding dNTP. The addition of methanol extract from 0.5×10^6 cells to the dADP, dGDP, dTDP and dCDP standards increases the relative incorporations to 9.8, 12.0, 15.3 and 11.2% respectively. Conversion of dNDP into dNTP and subsequent incorporation into polymer is stimulated by extracts of K562 cells. However, the extent of conversion is small, and incorporation of radiolabel is $\frac{1}{2}$ –3-fold above baseline, a very modest amount when compared with incorporation of dNTP standard. By comparison, as reported previously (North *et al.*, 1980), methanol extracts of HeLa cells contain substantial levels of nucleoside diphosphokinase activity.

In Fig. 5, dADP standards are incubated with various amounts of HeLa-cell extract representing between 0.055×10^6 and 0.22×10^6 cells. The extract of 0.22×10^6 cells converts 88% of the dADP into dATP, with subsequent incorporation into polymer. The extent of conversion decreases to 77 and 60% with extracts of 0.11×10^6 and 0.055×10^6 cells

Fig. 5. Effect of methanol extracts of HeLa cells on the incorporation of dADP into poly[d(A-T)]
O, dATP; ●, dADP; △, ▲ and ▽, dADP + HeLa cell extracts (△, 0.055×10^6 cells; ▲, 0.11×10^6 cells; ▽, 0.22×10^6 cells).

respectively. Thus, at a 10-fold lower concentration of extract than used with K562 cells, HeLa-cell extracts convert approx. five times more dADP into dATP than do K562 extracts.

The conversion is not limited to adenosine deoxynucleotides. The effect of adding HeLa-cell extract, representing 0.4×10^6 cells, to the four common dNDP species is shown in Fig. 6. dADP, dGDP, and dTDP are converted into their respective dNTP species and polymerized. Only the phosphorylation of dCDP does not occur at a rapid rate. All four dNMP species are poorly converted into dNTP species by extracts of both HeLa and K562 cells. Extracts of red blood cells had no detectable ability to phosphorylate dNMP and dNDP species as measured in our assay system. Peripheral lymphocytes, granulocytes and several other cell lines exhibited less kinase activity than K562 cells.

In our laboratory, the two-step methanol/ HClO_4 extraction procedure suggested by North *et al.* (1980) to eliminate contaminating enzymic activities has serious disadvantages. Radiolabelled dNTP species were added to lymphocyte pellets or to blank tubes, then extracted with 60% methanol or with 60% methanol followed by 0.5M- HClO_4 . Portions were analysed by high-pressure liquid chromatography, a gradient able to separate deoxynucleos-

Fig. 6. Effect of methanol extracts of HeLa cells on incorporation of dNDP into polymer
O, dNTP; ●, dNDP; △, dNDP + extract of 4×10^5 HeLa cells. Deoxynucleotides in (a)–(d) are as in Fig. 4.

ides and the deoxynucleotides being used. There is both lower and variable recovery of dNTP species (no cells: one-step, 100%; two-step, 90%; with cells: one-step, 95–99%; two-step, 55–92%), owing to the additional handling required in the two-step procedure and breakdown of dNTP species to nucleosides, mono- and di-phosphorylated forms.

Discussion

We have modified enzymic methods for the measurement of dNTP species in mammalian cells to provide the high degree of sensitivity required for the oftentimes small numbers of cells available for study. Detection of 0.1 pmol of dATP, dGTP, dTTP and 1.0 pmol of dCTP is possible. Assays are linear with respect to number of cells extracted [for lymphocytes and leukaemic- or transformed-cell lines (2.5×10^6 – 1.5×10^7 cells) and for red cells (0.1×10^9 – 1.5×10^9 cells)] and amount of extract added to the reaction mixture. We have examined whether contaminating deoxynucleotide-interconverting enzymes in simple methanol extracts affect the utility of this extraction procedure with regard to quantification of dNTP species. In our laboratory, methanol/HClO₄ extraction results in lower recovery of exogenously added dNTP. This loss of dNTP is sufficient to explain the 40% lower dNTP levels measured in two-step extracts of lymphocytes compared with those measured in simple single-step methanol extracts. North *et al.* (1980) report that dNTP levels determined from HeLa cells extracted by the two-step method are less than 20% of those reported by others using methanol extractions. They hypothesized that measurements on methanol extracts are seriously compromised by the presence of nucleases and deoxynucleotide-interconverting enzymes. Under these circumstances mono- and di-phosphates could be converted into triphosphates, and the deoxynucleotide polymer used as template could be degraded to supply monophosphates. The result would be artificially high estimates of the concentrations of intracellular deoxynucleotides. With our assay system, we have measured amounts of dATP, dGTP, dTTP and dCTP in methanol extracts of HeLa cells. The values of dGTP, dTTP, and dCTP are quite close to those of North *et al.* However, our dATP level was 3-fold lower, rather than higher, than theirs. Whether these data reflect differences between the assay systems or between the preparations of HeLa cells is uncertain. We could confirm the presence of significant amounts of deoxynucleotide-interconverting enzymes in methanol extracts of HeLa cells.

The major and significant differences between HeLa-cell extracts and those of normal peripheral blood cells, leukaemic cells and lymphoblast cell lines with respect to phosphorylating-enzyme activi-

ties point out the need for care in extrapolation of methods and results from one cell type to another. In peripheral blood cells and lymphoid cell lines in culture, 60%-methanol extracts have little ability to convert the dNDP into dNTP. The combination of low levels of dNDP species present in cell extracts and very low phosphorylating activities means that use of 60%-methanol extracts to assay dNTP does not result in 'artefactually' high values. More complex extraction procedures such as methanol/HClO₄ suffer from low yield and deoxynucleotide degradation, but may be required when extracts from certain types of cells are assayed.

Two different DNA polymerases have been used to assay dNTP species in the presence of primer. DNA polymerase I from *E. coli* was used by Solter & Handschumacher (1969). Some investigators have used DNA polymerase from *M. luteus*. This enzyme, when highly purified, has less nuclease activity than the polymerase from *E. coli* (Zimmerman, 1966), but is not widely available commercially. Substrate affinities of the two polymerases are similar (Zimmerman, 1966). The use of *M. luteus* DNA polymerase is advantageous, as background incorporation in the absence of limiting dNTP is three to six times lower than with *E. coli* DNA polymerase. For maximal sensitivity of assay, the *M. luteus* polymerase should be employed.

Investigators should be aware of the variable quality of the commercial reagents required to assay dNTP species. These are significant differences among various lots of polymerase and polymers. For example, during the past year we have purchased several lots of poly[d(I-C)] and *M. luteus* polymerase from two companies. Two of four lots of polymers and one of two lots of polymerase were unsatisfactory. The background incorporation of [³H]dNTP in the absence of one dNTP was high and standard curves were not linear. One lot of commercially prepared nuclease-treated DNA was unsatisfactory for the same reasons, and we have generally prepared this ingredient in our own laboratory.

Choice of an appropriate assay system depends on the level of sensitivity desired, the number of different dNTP species to be assayed routinely and the number of ingredients to be stocked. Maximal sensitivity is obtained with *M. luteus* rather than *E. coli* polymerase, regardless of the dNTP. Similarly, maximal sensitivity for assay of dATP, dGTP and dTTP is obtained with the appropriate synthetic alternating co-polymer rather than with nuclease-treated DNA as primer-template. Nuclease-treated DNA is likely to give a more satisfactory assay of dCTP, although some lots of poly[d(I-C)] can be used. If one or a base-pair of dNTP species is to be assayed routinely, then synthetic polymers are convenient to use. It is not convenient, however, to

test and stock the four different [^3H]dNTP species and two synthetic primer-templates necessary to assay all four dNTP species at maximal sensitivity. As a practical matter, if specimens contain more than 3 pmol of dNTP then we recommend that investigators consider utilizing *M. luteus* polymerase and nuclease-treated DNA for assay of all dNTP species. Under these conditions, only two [^3H]dNTP species and one primer-template need be available.

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