Compartmentation of guanine nucleotide precursors for DNA synthesis

Binh Trong NGUYEN and Wolfgang SADÉE*

Departments of Pharmacy and Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA 94143, U.S.A.

We have studied the kinetics of guanine incorporation into DNA in mouse T-lymphoma (S-49) mutant cells [PNPase (purine-nucleoside phosphorylase)- and HGPRTase (hypoxanthine: guanine phosphoribosyltransferase)-deficient] that are incapable of converting dGuo (deoxyguanosine) to Gua (guanine) ribonucleotides. Of the two possible pathways for an exogenous guanine source to reach DNA, firstly:

and secondly:

only the second pathway was found to be functional in providing guanine for DNA replication, although deoxyguanosine readily produced toxic cellular dGTP levels via the first pathway. The functional guanine-nucleotide-precursor pools for DNA are rather small; further, the depletion of the small GMP pool, but not that of GDP, GTP and dGTP, correlated well with the inhibition of DNA synthesis by mycophenolic acid, an IMP dehydrogenase inhibitor. These results support the hypothesis that guanine-nucleotide incorporation into DNA is highly compartmentalized and that a small functional guanine-nucleotide pool, e.g., the GMP pool, may serve a crucial role in limiting the availability of DNA precursor substrate.

INTRODUCTION

We have previously shown that guanine depletion by inhibition of IMP dehydrogenase leads to a drastic inhibition of DNA synthesis before GTP and dGTP pools are fully exhausted (Cohen *et al.*, 1981). Moreover, selective repletion of the dGTP pool with dGuo in HGPRTase-deficient lymphoma cells failed to restore DNA synthesis, and only a supply of Gua ribonucleotides in cells capable of salvaging exogenous Gua reversed the DNA-synthesis inhibition (Cohen *et al.*, 1981; Nguyen *et al.*, 1983). These paradoxical findings can be resolved if one postulates that the cellular dGTP pools have virtually no access to DNA replication, but that guanine must be recruited from the ribonucleotide precursor pools, e.g., GDP or GMP, to yield a small functional dGTP DNA-precursor pool. Increasing evidence points to the existence of a multienzyme channel (DNA replitase) that utilizes distant precursors to produce high levels of dNTPs in the immediate vicinity of the replication fork (veer Reddy & Mathews, 1978; Taheri *et al.*, 1981; Noguchi *et al.*, 1983). The hypothesis that Gua-DNA precursor biosynthesis represents an extreme case of metabolic compartmentation or channelling is tested here by selectively labelling dGTP via the 'de novo' and salvage pathways.

The formation of guanine nucleotides via salvage and 'de novo' pathways in mammalian cells is shown in

Scheme 1. 'De novo' pathway and salvage pathways of Guanine nucleotide synthesis

'De novo' pathway: IMP-DH, EC 1.2.1.14. Salvage pathways: PNPase, EC 2.4.2.1; HGPRTase, EC 2.4.2.8; dCK (deoxycytidine kinase), EC 2.7.1.74; RR (ribonucleotide 5'-diphosphate reductase), EC 1.17.4.1. MA blocks guanine formation *de novo* by inhibiting IMP-DH.

Abbreviations used: PNPase, purine-nucleoside phosphorylase; HGPRTase, hypoxanthine: guanine phosphoribosyltransferase; Guo, guanosine; dGuo, deoxyguanosine; Gua, guanine; MA, mycophenolic acid; Urd, uridine; Thd, ribosylthymine; dCyd, deoxycytidine; IMP-DH, inosinate dehydrogenase.

* To whom correspondence and reprint requests should be addressed.

Scheme 1. Both Guo and dGuo can provide cellular Gua ribonucleotides via Gua as the intermediate, whereas only dGuo is directly phosphorylated to Gua deoxyribonucleotides (Carson *et al.*, 1977; Gudas *et al.*, 1978). Hence the incorporation of dGuo into DNA can occur via two pathways, either direct ($\text{dGuo} \rightarrow \text{Gua} \rightarrow \text{GMP} \rightarrow \text{dGDP} \rightarrow \text{dGTP}$) or via Gua and its ribonucleotides with the involvement of ribonucleotide reductase ($\text{dGuo} \rightarrow \text{Gua} \rightarrow \text{GMP} \rightarrow \text{GDP} \rightarrow \text{dGDP} \rightarrow \text{dGTP}$). The latter pathway depends on the salvage enzymes HGPRTase and PNPase (Scheme 1). Therefore the use of either HGPRTase- or PNPase-deficient mutants should allow one to label dGTP selectively via only the kinase pathway, $\text{dGuo} \rightarrow \text{dGMP} \rightarrow \text{dGDP} \rightarrow \text{dGTP}$. The results with mouse T-lymphoma S-49 mutant cells (Gudas *et al.*, 1978; Ullman *et al.*, 1979) strongly suggest that only the pathway via Gua ribonucleotides and involving ribonucleotide reductase is capable of the efficient delivery of Gua into DNA.

MATERIALS AND METHODS

Mycophenolic acid (MA) was obtained from Eli Lilly and Co., Indianapolis, IN, U.S.A. All nucleotide reagents were purchased from Sigma, St. Louis, MO, U.S.A. [$8\text{-}^{14}\text{C}$]Gua (54.5 mCi/mmol), [$8\text{-}^3\text{H}$]dGuo (7.8 Ci/mmol) and [$8\text{-}^3\text{H}$]Guo (17 Ci/mmol) were purchased from ICN Pharmaceuticals, Irvine, CA, U.S.A. [$8\text{-}^{14}\text{C}$]dGuo (40 mCi/mmol) was purchased from Schwarz-Mann, Spring Valley, NY, U.S.A. [$8\text{-}^{14}\text{C}$]dGuo was used in some experiments to account for any potential ^3H exchange within the cells or during analysis; none was, however, observed. Moreover, the stability of the ^3H label of [$8\text{-}^3\text{H}$]dGuo was confirmed under the conditions of DNA and RNA separation (exposure to HClO_4 and KOH as discussed below) by subsequent reverse-phase h.p.l.c. analysis of the products.

Cell density was counted on a model Z_r Coulter counter (Coulter Electronics, Hialeah, FL, U.S.A.). Radioactive analysis was performed on a Beckman 9000LS liquid-scintillation counter (Beckman Instruments, Palo Alto, CA, U.S.A.). H.p.l.c. was performed on model-6000A solvent delivery system and model-660 solvent programmer (Waters Associates, Milford, MA, U.S.A.). The separation of dGTP and GTP and their di- and mono-phosphates was accomplished with a Partisil 10-SAX column (25 cm $\times$ 4.6 mm; Whatman Inc., Clifton, NJ, U.S.A.) (Pogolotti & Santi, 1982) and a linear elution gradient of 0.02 M- $\text{NH}_4\text{H}_2\text{PO}_4$ in 0.01 M-KCl, pH 3.6 (buffer A) and 1.0 M- $\text{NH}_4\text{H}_2\text{PO}_4$ in 0.5 M-KCl, pH 3.6 (buffer B) (buffer A to 100% buffer B for 20 min at a flow rate of 2 ml/min). This gradient provided superior separations of the deoxyribo- and ribo-nucleotides. For the assay of GMP and dGTP after guanine-nucleotide depletion with MA, the corresponding u.v. peaks were collected and rechromatographed isocratically with 0.1 M- and 0.8 M- $\text{NH}_4\text{H}_2\text{PO}_4$ respectively to increase the assay sensitivity and provide a second measurement of the GMP and dGTP pools. Fractions of sufficiently small volume (~ 0.3 ml) were collected to separate ^3H radioactivity associated with GTP and dGTP. Quantitative determinations of GMP, GDP, GTP and dGTP were performed with the use of external calibration curves after extraction of between 10^6 cells (for GTP) and 5×10^7 cells (for complete analysis of GMP, GDP, GTP and dGTP). Gua and dGuo were separated by h.p.l.c. on a

reverse-phase C₁₈ column (25 cm $\times$ 4.6 mm; Alltech Associates, Deerfield, IL, U.S.A.) and a 0.01 M- $\text{NH}_4\text{H}_2\text{PO}_4$ (pH 3.6) eluent buffer with 1% acetonitrile at a flow rate of 2.0 ml/min. Eluted compounds were detected at 254 nm with the use of a u.v. detector (model LC-15; Perkin-Elmer, Norwalk, CT, U.S.A.). The extraction of the cells with HClO_4 was described previously (Maybaum *et al.*, 1980; Cohen *et al.*, 1981). The quantitative detection limit for GMP and dGTP was ~ 0.1 nmol/ 10^8 cells (includes rechromatography of eluted peaks). The detection limit for dGuo by reverse-phase h.p.l.c. was ~ 0.1 nmol/ 10^8 cells. The pools of GMP (0.5 nmol/ 10^8 cells), GDP (4.5 nmol/ 10^8 cells) GTP (24 nmol/ 10^8 cells) and dGTP (0.8 nmol/ 10^8 cells) obtained by the Partisil SAX assay were similar to those previously determined with a multistep h.p.l.c. assay (Cohen *et al.*, 1981).

Mouse T-lymphoma (S-49) wild-type and mutant cells (HGPRTase-deficient and PNPase-deficient) were kindly provided by Dr. David W. Martin, Jr. (Genentech, South San Francisco, CA, U.S.A.). The HGPRTase-deficient clone S49 6MPR-3-3 and the PNPase-deficient clone S49 NSU-1 were used (Gudas *et al.*, 1978; Ullman *et al.*, 1979). The cells were grown at 37 °C in Dulbecco's modified Eagle's medium containing 10% inactivated horse serum under an atmosphere of CO₂/air (1:9) as described elsewhere (Gudas *et al.*, 1978; Ullman *et al.*, 1979; Cohen *et al.*, 1981). S-49 cells grow exponentially until a cell density of $\sim 2 \times 10^6$ /ml is reached. Cells that were grown exponentially, harvested by centrifugation and resuspended at a density below 10^7 cells/ml continue to produce DNA over several hours at a rate per cell similar to that observed in the exponential growth phase. For measurements of the smaller nucleotide pools (e.g. GMP and dGTP), several experiments were thus carried out at density of 5×10^6 cells/ml; there appeared to be little change in tracer uptake kinetics into GTP, RNA and DNA at different cell densities over a 3 h time period.

Cell labelling, extraction and the separation of RNA and DNA were carried out as described previously (Cohen *et al.*, 1981; Maybaum *et al.*, 1981). Control experiments with [^3H]Urd and [^3H]Thd confirmed the effective separation of the acid-precipitable ^3H radioactivity into RNA and DNA.

RESULTS

Tracer incorporation into RNA and DNA of S-49 wild-type and mutant cells

In order to test the pathways depicted in Scheme 1 in S-49 wild-type and in HGPRTase-deficient and PNPase-deficient mutants, incubations were carried out with [^3H]Guo, [^{14}C]Gua and [^3H]dGuo (Table 1). Independent experiments on the time course of tracer incorporation into DNA and RNA (see also the results in Figs. 2 and 3 below; RNA-uptake results not shown) revealed that a linear tracer incorporation is established with minimal delay (< 10 min) and maintained over at least 2–3 h. Therefore the results in Table 1 accurately reflect the efficiency of each tracer in labelling DNA and RNA in the three cell lines.

Guo and dGuo were more efficient in labelling the wild-type cells than Gua, possibly because of facilitated nucleoside transport across the cell membrane. The similar incorporation of [^3H]Guo and [^3H]dGuo into

Table 1. Labelling of DNA and RNA of S-49 cells with [³H]Gua, [¹⁴C]Gua and [³H]dGua (~ 10⁶ cells/ml; 10 ml incubation mixtures; 1 µCi per sample over a 180 min incubation period)

Wild-type, HGPRTase- and PNPase-deficient cells were used. Results are expressed as d.p.m./30 min per 10⁸ cells recovered in the DNA and RNA fractions, and as means of duplicate measurements in two dependent experiments with s.d. usually below 10%. The RNA/DNA c.p.m. ratios were subject to some variability from one experiment to the next. Repeat experiments gave the following RNA/DNA d.p.m. ratios: [¹⁴C]Gua in PNPase-deficient cells, 4.58 ± 0.74 ; [³H]dGua in HGPRTase-deficient cells, 1.134 ± 0.19 ; [³H]dGua in PNPase-deficient cells, 0.97 ± 0.12 ($\pm$ s.d., $n = 4$).

	Labelling (d.p.m./30 min per 10 ⁸ cells)					
	[³ H]Gua		[¹⁴ C]Gua		[³ H]dGua	
	RNA	DNA	RNA	DNA	RNA	DNA
Wild-type	289 800	37 800	44 700	11 500	266 000	46 700
HGPRTase-deficient	6000	n.m.*	n.m.*	n.m.*	11 300	8600
PNPase-deficient	10000	1300	64 100	12 500	16 500	16 800

*Not accurately measurable because of background noise (signal-to-noise ratio ≤ 3 ; background noise was determined by parallel incubations at 0 °C).

Fig. 1. Anion-exchange chromatography on Parisil 10-SAX of the acid-soluble fraction of (a) PNPase- and (b) HGPRTase-deficient mutant cells after labelling with [³H]dGua (10 µCi/100 ml; ~ 10⁶ cells/ml; over 3 h)

Gua and dGua were not separated under these assay conditions. A small amount of ³H radioactivity also appeared in ATP (largest h.p.l.c. peak eluted before GTP); however, because of the extensive tracer dilution, no interference by ³H-labelled adenine nucleotides with the interpretation of RNA and DNA tracer-incorporation data is expected. The actual number of d.p.m. in GTP, incorporated via the 'deficient' pathway, was quite variable and sensitive to the incubation conditions.

RNA and DNA suggests that dGua is predominantly converted into Gua by the wild-type cells before conversion into nucleotides. Further, the much lower labelling efficiency of [¹⁴C]Gua for RNA and DNA in the HGPRTase-deficient cells and of [³H]dGua for RNA in the HGPRTase- and PNPase-deficient cells confirmed the presence of severe deficiencies of HGPRTase and PNPase respectively. The rather low labelling efficiency of [³H]Gua in both mutants confirms the lack of any significant Guo kinase activity, which is common to mammalian cell lines. However, the HGPRTase and PNPase deficiencies in the mutant cells are not absolute; hence, metabolic fluxes via these pathways are still

measurable (Table 1). The culture medium with 10% horse serum displayed some PNPase activity against [³H]dGua (40% conversion into [³H]Gua over 4 h in the medium itself); however, a 2–4-fold dilution of the horse serum, which reduced [³H]Gua formation accordingly, did not change the DNA/RNA labelling ratio as an indicator of tracer flux into ribo- and deoxyribo-nucleotide pools. Therefore residual PNPase activity was present within the PNPase-deficient mutant cells.

H.p.l.c. analysis of cellular Gua nucleotide pools

The chromatograms in Fig. 1 reveal preferential labelling of dGTP over GTP with [³H]dGua in

Fig. 2. Incorporation and subsequent elimination of dGuo tracer in S-49 mutant cells

(a) Incorporation of [^{14}C]dGuo ($0.5 \mu\text{Ci}/10 \text{ ml}$; 2×10^6 cells/ml) into the cellular dGTP pool and DNA of PNPase-deficient mutants. (b) Elimination of ^{14}C radioactivity from DNA and cellular dGuo and dGTP pools after removal of the [^{14}C]dGuo tracer by centrifugation and resuspension of the cell pellet in tracer-free medium. Cells were labelled for 4 h under conditions similar to those in (c). (c) Time course of ^3H radioactivity incorporation into DNA and the cellular dGTP pool of HGPRTase-deficient cells after removal of the [^3H]dGuo tracer from the medium. Cells were labelled at $2.8 \mu\text{Ci}/10 \text{ ml}$ of medium over 3 h at an initial density of 2×10^6 cells/ml. ^3H radioactivity in dGMP, dGDP and dGTP was combined and is presented as dGXP; most of the activity was associated with dGTP, and ^3H radioactivity in dGuo and Gua ribonucleotides was negligible. Since the dGuo tracers were used in concentrations that do not measurably affect cellular pool sizes, the d.p.m. values are directly proportional to the specific radioactivities of the Gua-nucleotide pools.

PNPase-deficient and HGPRTase-deficient mutants. The labelling of dGTP and GTP was time-dependent, with different equilibration times for both pools in the two mutants, but dGTP was more intensely labelled at all time points between 0 and 4 h after tracer addition. Given the much larger pool size of GTP, the specific-radioactivity ratio dGTP/GTP is very large after a 3 h labelling period. A large ^3H peak occurred where Gua and dGuo are co-eluted on SAX columns (Fig. 1). Reverse-phase h.p.l.c. of this eluate fraction to separate Gua and dGuo revealed that, in PNPase-deficient mutants, $\sim 40\%$ of the ^3H radioactivity was associated with intact [^3H]dGuo, whereas only 1–2% of the ^3H radioactivity was accounted for by [^3H]dGuo in the HGPRTase-deficient mutant, with most of the ^3H in the Gua peak. Cellular dGuo was present below detectable levels by u.v. analysis in the mutant cells ($< 0.1 \text{ nmol}/10^8$ cells).

Incorporation of dGuo tracer into dGTP and DNA

The time course of intracellular labelling with dGuo tracer is shown in Fig. 2. The PNPase-deficient S-49 cells were used (Figs. 2a) and 2b), [^{14}C]dGuo), whereas Fig. 2(c) ([^3H]dGuo) shows the results with HGPRTase-deficient mutants. Note that the ^{14}C radioactivity in dGTP rises slowly, whereas DNA synthesis is established at a constant rate immediately after tracer addition (Fig. 2a). Upon tracer removal from the medium, net ^{14}C incorporation into DNA ceased without delay (Fig. 2b), although ^{14}C activity in dGTP remained at high levels for at least two hours. The persistence of [^{14}C]dGuo in the PNPase-deficient mutant prevents the evaluation of dGTP turnover, since dGuo and dGTP are metabolically interconvertible. Therefore the dGTP pool was also labelled with [^3H]dGuo in HGPRTase-deficient mutants (Fig. 2c) in order to rapidly metabolize the intracellular dGuo to Gua as documented above. After removal of the tracer from the medium after a 3 h labelling period, the

decline of ^3H in the dGMP, dGDP and dGTP pools (combined as dGXP after separate collection of the corresponding h.p.l.c. peaks) was at least biphasic, but the rate of tracer removal was rather slow, and measurable ^3H radioactivity was retained for over 17 h (Fig. 2c).

Incorporation of guanine tracer into nucleotides and DNA

Upon incubation of S-49 wild-type cells with [^{14}C]Gua, the ^{14}C radioactivity in GMP, GDP and GTP rises until it approaches steady state in the GTP pool after approx. 2 h (Fig. 3a). In contrast, a linear DNA incorporation rate is reached within less than 10 min; the delay time in repeat experiments was usually at or below 5 min. Similar results were obtained with the PNPase-negative mutants under various tracer concentrations and cell densities (results not shown). The ^{14}C radioactivity in the dGTP pool was too low to be detectable under all [^{14}C]Gua labelling conditions.

Competition for DNA uptake between dGuo and Gua

With the use of the PNPase-deficient cells, one can test the competition between increasing doses of [^{14}C]Gua and [^3H]dGuo for their DNA utilization. As observed previously with the HGPRTase-deficient cells (Cohen *et al.*, 1981), dGuo ($2\text{--}100 \mu\text{M}$) was unable to restore any DNA synthesis or cell growth in PNPase-deficient cells treated with $1 \mu\text{M}$ -MA, whereas maximum rescue of DNA synthesis and cell growth to $\sim 80\%$ of control was achieved with $300\text{--}400 \mu\text{M}$ -Gua. Further, subtoxic levels of dGuo failed to affect [^{14}C]Gua tracer uptake into DNA under a variety of conditions [$\pm$ MA, $\pm$ unlabelled Gua (up to $400 \mu\text{M}$)]. It therefore appeared that the dGTP pool derived from dGuo was not utilized for DNA synthesis. However, these low subtoxic levels caused only minimal elevations (< 2 -fold) of the cellular dGTP pool measured at 4 h after dGuo addition. The 50%-growth-inhibitory

Fig. 3. [^{14}C]Gua incorporation into GTP, GDP, GMP, and DNA

Wild-type S-49 cells were labelled with $1\ \mu\text{Ci}$ of [^{14}C]Gua in 10 ml at an initial cell density of 6.2×10^6 cells/l. The ^{14}C radioactivity in the dGTP pool was too low for quantitative analysis. Results with PNPase-deficient mutants were similar (results not shown).

concentrations of dGuo (IC_{50}) were $30\ \mu\text{M}$, $12\ \mu\text{M}$ and $30\ \text{mM}$ in wild-type and in PNPase-deficient and HGPRTase-deficient mutants respectively.

We then decided to determine whether a drastically increased dGTP pool, derived from dGuo, could force its utilization for DNA synthesis in preference over guanine supply via the ribonucleotide reductase pathway. As elevated dGTP pools cause the selective depletion of dGTP and dTTP (Ullman *et al.*, 1979), dCyd was employed to bypass dGTP toxicity. However, dCyd also shares a common activation pathway with dGuo, i.e. dCyd kinase (Scheme 1), for which it was reported to have two-orders-of-magnitude higher affinity than dGuo (Gower *et al.*, 1979; Ives & Durham, 1970). We therefore studied first the effects of dCyd on [^3H]dGuo uptake and metabolism in PNPase-deficient cells. Concentrations of dCyd between 2.5 and $5\ \mu\text{M}$ maximally reduced [^3H]dGuo tracer uptake into dGTP and DNA to $\sim 20\%$ of control, and even much higher dCyd concentrations failed to depress [^3H]dGuo utilization further. Therefore a small dCyd-insensitive pathway of $\text{dGuo} \rightarrow \text{dGMP}$ appeared to exist.

A series of further experiments was then carried out in PNPase-deficient cells to establish concentrations of a combination of MA (to block GMP synthesis *de novo*), Gua, dGuo, and dCyd to maintain GTP levels and DNA synthesis close to control values while drastically increasing dGTP levels. A combination of $10\ \mu\text{M}$ -MA, $400\ \mu\text{M}$ -Gua, $500\ \mu\text{M}$ -dGuo and $5\ \mu\text{M}$ -dCyd allowed $\sim 60\%$ of control [^3H]dThd incorporation into DNA and established pools of 60 ± 4.2 and $14 \pm 2\ \text{nmol}/10^8$ cells

($n = 4$) for GTP and dGTP respectively, determined after a 4 h incubation period. Under these conditions the use of [^{14}C]Gua and [^3H]dGuo ($4\ \mu\text{Ci}$ of each tracer/40 ml of incubation medium; 3.7×10^6 cells/ml in separate incubations over 4 h revealed a total DNA-synthesis rate of $\sim 7\ \text{nmol}$ of dG/10⁸ cells per 30 min, after correction for the specific radioactivities in the medium and assuming no further [^3H]tracer dilution within the cells. Despite the drastic increase of the dGTP pool derived from dGuo, [^{14}C]Gua incorporation via ribonucleotide reduction still accounted for more guanine bases incorporated into DNA than did [^3H]dGuo via the direct kinase pathway (50–70% from [^{14}C]Gua as against 30–50% from [^3H]dGuo). However, the leakage of [^3H]dGuo into Gua and its ribonucleotides, caused by residual PNPase activity, prevents a precise quantification of the fluxes along the two pathways under these conditions.

Turnover rate of guanine-nucleotide pools

Studies on the turnover rate of nucleotide pools were performed after labelling wild-type and PNPase-deficient cells with [^{14}C]Gua. A representative experiment with PNPase-deficient cells is shown in Fig. 4. Approx. 20–40 min after tracer removal from the medium, accumulation of ^{14}C radioactivity in DNA ceased while ^{14}C radioactivities in the cellular GTP pool decreased rather slowly. Furthermore, the ^{14}C radioactivity in the GDP pool remained nearly constant, whereas that in GMP initially decreased rapidly to 35% at 15 min after tracer removal and to $\sim 25\%$ thereafter (Fig. 5). In two independent experiments with smaller incubation volumes (1 ml) that permit a more rapid extraction of the cell pellets and thereby could suppress any factitious

Fig. 4. Time course of elimination of ^{14}C radioactivity in the GTP, GDP, GMP, and DNA pools of PNPase-deficient S-49 cells after a 150 min labelling period with $1\ \mu\text{Ci}$ of [^{14}C]Gua and transfer of the cells into tracer-free medium (initial cell density 1.5×10^6 cells/ml; 10 ml incubation mixtures)

Fig. 5. Effect of MA on Gua-nucleotide pools and DNA synthesis in S-49 wild type cells (100 ml incubation mixtures; 3 h; initial cell density, 10^6 cells/ml)

The h.p.l.c. assay was unable to detect GMP pools at or below 20% of the control value (0.5 nmol/ 10^8 cells). Relative DNA-synthesis rates were determined at 3 h by observing [3 H]Thd incorporation into DNA (1 ml incubation mixtures; 20 min).

conversion among the Gua ribonucleotides, the 14 C radioactivity in GMP (~ 1000 d.p.m.) decreased to approx. 10% within 10 min of the tracer removal and remained at 5–15% of control for at least 1 h.

In order to determine the relationship between MA-induced inhibition of DNA synthesis and the depletion of individual guanine-nucleotide pools, [3 H]dThd incorporation into DNA and pools of GMP, GDP, GTP and dGTP were measured 3 h after addition of the drug (MA) (Fig. 5). Whereas the levels of GDP, GTP and dGTP were reduced by 50–70% by 0.5–1.0 μ M MA, [3 H]Thd incorporation into both the DNA and the GMP pool was largely reduced or abolished by 0.2–0.4 μ M-MA.

DISCUSSION

Cellular dGTP pool: feedback inhibition of ribonucleotide reductase, but ineffective utilization for DNA

Preliminary experiments with Guo, Gua and dGuo tracers established that the PNPase- and HGPRTase-deficient mutants of mouse T-lymphoma S-49 cells can serve as tools to label selectively the cellular dGTP pool and, therefore, to study its access to DNA (Table 1, Fig. 1).

Because of the small size of the cellular dGTP pool and the large demand for DNA synthesis, one would expect a rapid turnover rate for dGTP if this cellular pool served as the DNA-precursor pool. The turnover rate of the cellular dGTP pool after labelling with [3 H]dGuo was studied in the HGPRTase-deficient mutants, since these mutants were capable of rapidly metabolizing residual tracer in the cell by the action of PNPase. The $t_{1/2}$ of 3 H

elimination from the dGTP pool, after washing out the tracer from the HGPRTase-deficient mutants, was at least biphasic and in the range 2–4 h (Fig. 2c). This value range can now serve as a measure of the dGTP turnover rate; applying the equation:

$$\text{Turnover rate} = (0.693/t_{1/2}) \cdot (\text{pool size})$$

yields a turn-over rate of less than 0.1 nmol/30 min per 10^8 (the dGTP pool size is ~ 0.8 nmol/ 10^8 cells). In contrast, the actual DNA synthesis rate in S-49 cells reaches 8–9 nmol of dGuo/30 min per 10^8 cells (Maybaum *et al.*, 1981), which suggests that the labelled cellular dGTP pool does not function as the Gua-DNA precursor pool. Moreover, whereas the dGuo tracer equilibrates very slowly with the dGTP pool, a constant rate of tracer incorporation into DNA is established within less than 5 min. These results imply the existence of an exceedingly small functional Gua-DNA precursor pool that is independent of the cellular dGTP pool and rapidly equilibrates with the exogenous dGuo tracer. The lack of substantial dGuo tracer incorporation into DNA cannot be explained by a lack of phosphorylation in S-phase, since dCyd kinase was found to be active in S-49 cells in S-phase (Forsdyke & Scott, 1980; Maybaum *et al.*, 1981). The curvilinear decline of 3 H radioactivity in dGTP (Fig. 2c; plotted on a log-linear scale) could result from slightly different turnover rates throughout the cell cycles. However, the dramatic discrepancy between dGTP turnover and dGuo demand for DNA synthesis cannot be resolved by such turnover-rate fluctuations throughout the cell cycle.

The preferential incorporation of dGuo into mitochondrial DNA could provide an alternative explanation for the discrepant labelling kinetics of dGTP and DNA. A high-affinity dGuo kinase was reported to exist in mitochondria (Gower *et al.*, 1979), so that the acid-precipitable radioactivity could represent mitochondrial DNA. However, after isolation of the mitochondria by centrifugation (van den Bogert *et al.*, 1983), only 20% or less of the total cellular 3 H radioactivity in DNA, after incubation of PNPase-deficient mutants with [3 H]dGuo, was accounted for by mitochondrial nucleic acid (results not shown).

Gudas *et al.* (1978) have provided convincing arguments that the cellular toxicity of dGuo in S-49 cells is caused by dGTP-mediated feedback inhibition of ribonucleotide reductase. They showed that both dCyd kinase (required for dGuo activation to dGMP) (Gower *et al.*, 1979)-deficient mutants and a mutant with impaired dGTP feedback regulation of ribonucleotide reductase (Eriksson *et al.*, 1981) are resistant to dGuo. Elevated dGTP pools resulting from dGuo salvage cause the selective depletion of dCTP and dTTP (Ullman *et al.*, 1979; Lowe & Grindey, 1976) as a result of feedback inhibition of ribonucleotide reductase. These results demonstrate that dGuo is effectively activated to dGTP, which has access to the regulatory unit of ribonucleotide reductase; yet the dGTP pool generated by this pathway has limited or no access to DNA replication, even though toxic dGTP levels are achieved.

Studies on the functional Gua-DNA precursor pool

In several mammalian cell lines the drastic DNA-synthesis inhibition caused by IMP-DH block is associated with the depletion of Gua ribonucleotides rather than dGTP (Cohen *et al.*, 1981; Nguyen *et al.*,

1983). Depletion of a discrete functional Gua ribonucleotide precursor pool may thus play a crucial role in limiting the substrate supply for DNA replication under conditions of purine starvation. Results with [^{14}C]Gua labelling point to GMP or a small Gua-nucleotide pool in rapid equilibrium with GMP for such a role. First, upon addition of [^{14}C]Gua to the medium, linear ^{14}C uptake into DNA is established within a few minutes, which suggests that the functional precursor pool for dGuo-DNA is rather small. Indeed, GMP ranks as the smallest ribonucleotide pool of the purines and pyrimidines (Maybaum *et al.*, 1980; Cohen *et al.*, 1980); certainly, the [^{14}C]Gua tracer does not appear to equilibrate with the cellular GDP and GTP pools before entering DNA (Fig. 3). Upon tracer removal from the medium, ^{14}C uptake into DNA ceases within 15–30 min, and GMP represents the only Gua-nucleotide pool analysed that also loses a large fraction of its ^{14}C label within 10 min. Moreover, upon inhibition of guanine nucleotide biosynthesis *de novo* by MA, the GMP pool is the only pool that is largely depleted when DNA synthesis ceases (Fig. 5). These results are consistent with previous findings that inhibition of IMP-DH by MA causes an immediate and dramatic inhibition of DNA synthesis before Gua-nucleotide pools, other than GMP, are exhausted (Cohen *et al.*, 1981; Ives & Durham, 1970).

Possible metabolic channelling of guanine into DNA

The postulated DNA replitase system (veer Reddy & Mathews, 1978; Taheri *et al.*, 1981; Noguchi *et al.*, 1983) provides one possible hypothesis consistent with these findings. This multienzyme complex includes nucleotide kinases, ribonucleotide reductase and DNA polymerase, among other enzymes, and it is thought to utilize ribonucleotide 5'-diphosphate precursors for DNA synthesis without allowing the intermediates to equilibrate with their respective cellular pools. Although the cellular dGTP pool has virtually no access to DNA (in contrast with the pyrimidine dNTPs, which apparently can bypass the replitase) (Forsdyke & Scott, 1980; Nicander & Reichard, 1983; Chiba *et al.*, 1984), it retains its ability to feedback-inhibit UDP and CDP ribonucleotide reductase, possibly by binding at the regulatory site on the *outside* of the putative multienzyme channel (as previously suggested by Scott & Forsdyke, 1980). The results reported here are consistent with compartmentation on the molecular scale (equivalent to channelling), rather than on the larger scale of the cell organelles. Although separate cytoplasmic and nuclear dNTP pools have been reported (Gudas *et al.*, 1978), our kinetic-tracer results suggest a considerably smaller functional Gua-DNA precursor pool than the dGTP pool that is expected to be present in the nuclei. Furthermore, the inability of deoxyribonucleosides to overcome hydroxy-

urea toxicity (caused by ribonucleotide reductase inhibition) is consistent with the exclusive uptake of DNA-Gua via the ribonucleotide pathway (Scott & Forsdyke, 1980).

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REFERENCES

- Carson, D. A., Kaye, J. & Seegmiller, J. E. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5677–5681
- Chiba, P., Bacon, P. E. & Cory, J. G. (1984) *Biochem. Biophys. Res. Commun.* **123**, 656–662
- Cohen, M. B., Maybaum, J. & Sadée, W. (1980) *J. Chromatogr.* **198**, 435–441
- Cohen, M. B., Maybaum, J. & Sadée, W. (1981) *J. Biol. Chem.* **256**, 8713–8717
- Eriksson, S., Gudas, L. J., Clift, S. M., Caras, I. W., Ullman, B. & Martin, D. W., Jr. (1981) *J. Biol. Chem.* **256**, 10193–10197
- Forsdyke, D. R. & Scott, F. W. (1980) in *Cell Compartmentation and Metabolic Channeling* (Nover, L., Lyuen, F. & Mothes, K., eds.), pp. 117–184, VEB Gustav Fischer Verlag, Jena, and Elsevier/North-Holland, Amsterdam
- Gower, W. R., Jr., Carr, M. C. & Ives, D. H. (1979) *J. Biol. Chem.* **254**, 2180–2183
- Gudas, L. J., Ullman, B., Cohen, A. & Martin, D. W., Jr. (1978) *Cell* **14**, 531–538
- Ives, D. H. & Durham, J. P. (1970) *J. Biol. Chem.* **245**, 2285–2294
- Lowe, J. K. & Grindey, G. B. (1976) *Mol. Pharmacol.* **12**, 177–184
- Maybaum, J., Klein, F. K. & Sadée, W. (1980) *J. Chromatogr.* **188**, 149–158
- Maybaum, J., Cohen, M. B. & Sadée, W. (1981) *J. Biol. Chem.* **256**, 2126–2130
- Nguyen, B. T., Cohen, M. B. & Sadée, W. (1983) *Cancer Chemother. Pharmacol.* **11**, 117–119
- Nicander, B. & Reichard, P. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1347–1351
- Noguchi, H., veer Reddy, G. P. & Pardee, A. B. (1983) *Cell* **32**, 443–541
- Pogolotti, A. L., Jr. & Santi, D. V. (1982) *Anal. Biochem.* **126**, 335–345
- Scott, F. W. & Forsdyke, D. R. (1980) *Biochem. J.* **190**, 721–730
- Taheri, M. R., Wickremasinghe, R. G. & Hoffbrand, A. B. (1981) *Biochem. J.* **194**, 451–461
- Ullman, B., Gudas, L. J., Clift, S. M. & Martin, D. W., Jr. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1074–1078
- van den Bogert, C., Dontje, B. H. J. & Kroon, A. M. (1983) *Cancer Res.* **43**, 2247–2251
- veer Reddy, G. P. & Mathews, C. K. (1978) *J. Biol. Chem.* **253**, 3461–3467

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