Mitochondrial deoxynucleoside triphosphates are formed and regulated by a network of anabolic and catabolic enzymes present both in mitochondria and the cytosol. Genetic deficiencies for enzymes of the network cause mitochondrial DNA depletion and disease. We investigate by isotope flow experiments the interrelation between mitochondrial and cytosolic deoxynucleotide pools as well as the contributions of the individual enzymes of the network to their maintenance. To study specifically the synthesis of dGTP used for the synthesis of mitochondrial and nuclear DNA, we labeled hamster CHO cells or individual enzymes of the network to cause mitochondrial DNA present both in mitochondria and the cytosol. Genetic deficiencies of enzymes in the network (3). In some cases the genetic deficiency leads to depletion of mt deoxyribonucleoside triphosphate (dNTP) (4–6), in other cases to overproduction (7–9). In both instances the disturbed normal balance between the four dNTPs results in disease. Early examples are the catabolic phosphorylases that degrade purine (7) or thymine nucleotides (9). Their loss causes overproduction of dGTP or dTTP, with the affected individuals suffering from immune deficiency (7, 8) or mitochondrial neurogastrointestinal encephalomyopathy (9), respectively. Later discovered examples are the anabolic mt salvage enzymes deoxyguanosine kinase (dGK) (4) or thymidine kinase 2 (TK2) (5) and more recently the cytosolic small subunit of ribonucleotide reductase p53R2 (6), all leading to mt DNA depletion in terminally differentiated cells.

mt DNA synthesis occurs independently of nuclear DNA replication (10), also outside S phase. Both nuclear and mt DNA synthesis require a balanced supply of the four dNTPs (11). In cycling cells these come largely from the de novo reduction of ribonucleotides by ribonucleotide reductase (12), a cytosolic enzyme. They must be transported through the inner mt membrane to reach the site of mt DNA synthesis. Ribonucleotide reductase consists of a complex between two proteins, R1 + R2 or R1 + p53R2. R2 is S phase-specific (13), and the canonical R1/R2 complex is the predominant form of the enzyme in dividing cells. During late mitosis R2, but not p53R2, is degraded (13), and in postmitotic cells R1/p53R2 catalyzes a low reduction of ribonucleotides providing dNTPs for mt DNA synthesis (14, 15). p53R2 was originally discovered as a p53-inducible protein (16) and believed to be involved in repair after DNA damage. Recent evidence (6, 14, 15) suggests that the main function of p53R2 is, instead, to provide dNTPs for mt DNA synthesis in nongrowing cells.

mt dNTPs are also formed by the salvage of deoxyribonucleosides catalyzed by four different deoxynucleoside kinases (1, 17). Two of them, thymidine kinase 1 (TK1) and deoxycytidine kinase (dCK) are in the cytosol, and their products must enter mitochondria to be available for mt DNA synthesis. The other two, TK2 and dGK, are localized inside the mitochondria. The substrate specificity of TK2 permits the phosphorylation of thymidine and deoxycytidine, and that of dGK permits the phosphorylation of deoxyguanosine and deoxyadenosine.
Guanine Deoxyribonucleotides and Mitochondrial DNA

Between themselves the two intra-mt kinases should therefore be able to produce all four canonical deoxyribonucleotides for mt DNA synthesis. This presupposes that the appropriate deoxynucleosides are available. Also the combined substrate specificities of the two cytosolic deoxynucleoside kinases provide the potential to salvage all four deoxynucleosides, with TK1 phosphorylating thymidine and dCK phosphorylating the other three deoxynucleosides.

A third group of enzymes catalyzes reactions involved in the catabolism of deoxyribonucleotides and has a regulatory function. Both in the cytosol and mitochondria, the 5'-deoxynucleotidases cdN and mdN (18) form substrate (futile) cycles together with deoxynucleoside kinases and make possible the diffusion of deoxynucleotides through the plasma membrane into the extracellular space (19). Other catabolic enzymes are nucleoside deaminases and phosphorylases (7, 9) that degrade deoxynucleosides to purine or pyrimidine bases. Thus an intrinsic network of anabolic and catabolic enzymes operates for the supply of both mt and nuclear dNTPs.

In our laboratory we investigate the sources of dNTPs for mt DNA synthesis in intact cells, especially the interplay between their de novo synthesis from ribonucleotides and their salvage from deoxynucleosides. In earlier work we defined the pathways that in human and mouse cultured cells provide mt dTTP (20, 21). We studied by isotope-chase experiments with radioactive thymidine the flow of metabolites and found that in cycling cells the R1/R2 ribonucleotide reductase and, to a minor extent, salvage of thymidine by TK1 were the major sources of the nucleotide. dTMP enters mitochondria (22) and is phosphorylated to dTTP by intra-mt kinases. In quiescent cells dTTP is synthesized by the R1/p53R2 ribonucleotide reductase in the cytosol (14, 15) and by salvage of thymidine by TK2 in mitochondria (23). In both cycling and quiescent cells the cytosolic and mt dTTP pools turn over rapidly and attain a dynamic equilibrium. In cycling cells the newly synthesized dTTP is largely incorporated into DNA, in quiescent cells there is a constant degradation and resynthesis by substrate cycles between thymidine kinases and 5'-deoxynucleotidases (21, 23).

Knowledge concerning the sources of the other three mt dNTPs is scarce. Having established the ground rules for the synthesis of dTTP, we here investigate the synthesis of dGTP, a purine dNTP, in cultured hamster CHO cells and in human fibroblasts by pulse-chase experiments with labeled deoxyguanosine. We found that the major part of mt dGTP is formed by ribonucleotide reduction in the cytosol and imported into mitochondria. In both cycling and resting cells devoid of dCK activity, isotopic GdR was phosphorylated by the mitochondrial dGK, and the labeled guanine deoxynucleotides were exported into the cytosol. In dCK proficient cells labeled GdR was also phosphorylated in the cytosol by dCK. The enzymes of the network rapidly established a dynamic equilibrium between guanine deoxynucleotides, resulting from their shuttling between the cytosol and mitochondria and their incorporation into DNA in cycling cells or their degradation in quiescent cells. This equilibrium was disturbed when ribonucleotide reduction was inhibited with hydroxyurea causing decreased turnover of cytosolic dGTP and accumulation of labeled dGTP in both cytosol and mitochondria.

EXPERIMENTAL PROCEDURES

Materials—[5-3H]deoxycytidine (9,000 cpm/pmol) and [8-3H]deoxyguanosine (9,000 cpm/pmol) were from Moravek (Brea, CA). The labeled nucleosides were used at 10–20-fold diluted specific radioactivity for measurements of enzyme activity. In experiments with whole cells [8-3H]deoxyguanosine was used without dilution. Ni2+-nitriloatriacetic-agarose for affinity purification was from Qiagen. Immucillin H was a gift from Dr. Vern Schramm (Yeshiva University, New York, NY). Aphidicolin, hydroxyurea, and digitonin were from Sigma-Aldrich. pET-9d vectors containing either the human dCK or dGK cDNA sequences in frame with a six-histidine tag were gifts from Dr. Staffan Eriksson (Swedish University of Agricultural Sciences, Uppsala, Sweden). Pro- dCK+ and Pro- dCK- CHO cell lines were gifts from Dr. Mark Meuth (University of Sheffield, Sheffield, UK). From both strains we prepared HGPRT- CHO cell lines by selection with 5 µg/ml of 6-thioguanine. A HGPRT- human Lesch-Nyhan fibroblast line (code FFF0141991) was from the cell bank of Gaslini Hospital (Genova, Italy).

Enzymes and Enzyme Assays—We prepared homogeneous dCK and dGK proteins with N-terminal histidine tags by procedures developed in the Eriksson laboratory (24, 25). The activities and substrate specificities for GdR and CdR of these preparations do not differ from those of the enzymes without the histidine tag (24, 25). We assayed the pure enzymes at different substrate concentrations by adsorption of the formed radioactive nucleotides on Whatman DE-81 filter discs as described (24, 25). By the same general procedures we determined in extracts from whole cells, mitochondria, or cytosol the activities of the native enzymes. We prepared the extracts by procedures used in our laboratory (26) and added glycerol to a final concentration of 25% before storing them at −80 °C. We determined the total protein concentration in the extracts (27) with crystalline bovine serum albumin as standard. All of the assays were run with two different aliquots of protein to check for proportionality of the reactions.

Cell Growth and Isotope Experiments—We grew CHO cells in α-minimal essential medium (Invitrogen) with 7% fetal calf serum (FCS) and human fibroblasts in Dulbecco's modified Eagle's medium (Invitrogen) with 10% FCS. In isotope experiments we used cycling CHO cells (50–70% in S phase determined by flow cytometry) at the second day after seeding 0.8 million cells/10-cm dish. We could not obtain quiescent CHO cells. In isotope experiments with the slowly growing cycling human fibroblasts (10–15% in S phase), we used the cells 3 days after plating 0.4 million cells/10-cm dish. To obtain quiescent cells (28), we continued the growth of fibroblasts with 10% FCS until confluence (10 days), maintained them with 0.1% FCS for 10–30 days with medium changes twice a week (≤1.5% in S phase), and then used them for the experiments. Isotope experiments measuring the metabolism of labeled deoxyguanosine were patterned on similar experiments with labeled thymidine (20). Two hours before the actual start of the experiment, we changed the medium in a 37 °C warm room to fresh medium with dialyzed FCS. Where indicated we then added 0.5 µM immucillin H (at 15 min), 10 µM aphidicolin (at 30 min), or 3
mm hydroxyurea (at 60 min) before the start of the labeling period with 1 μM [3H]GdR. In chase experiments we removed the labeled medium after the indicated time, replaced it with fresh prewarmed medium containing 1 μM nonlabeled GdR, and continued incubation. To terminate the experiments, we transferred the dishes to an ice bath in the cold room, removed the medium, and washed the cells four times with ice-cold phosphate-buffered saline. We scraped the cells, homogenized by pushing the suspension 10 times through a syringe, and separated by centrifugation nuclei + mitochondria from the cytosol as described (20, 21). Because CHO cells were more difficult to homogenize, we added 0.025 ml of digitonin (5 mg/ml) to 0.5 ml of cell suspension containing 0.5% bovine serum albumin, rapidly broke the cell membrane by five strokes in the syringe, and added 1 ml of the bovine serum albumin containing buffer to decrease the digitonin concentration. Digitonin at the chosen concentration permeabilizes the cell membrane and thereby facilitates cell homogenization without damaging the inner mt membrane (29). All of the manipulations were made rapidly in the cold, and cell breakage was controlled in a microscope. The separation of mt and cytosolic dNTP pools and their analyses were described earlier (20, 21).

Analytical Procedures—We used two independent methods to analyze the flow of isotope from deoxyguanosine into DNA via mt and cytosolic deoxyguanosine phosphate pools. In one method we measured at different time intervals the total amount of isotope present in the pools, in the other we determined the specific radioactivity of dGTP. The first method relied on the separation of the deoxynucleotides by HPLC and measured the radioactivity in the separated nucleotides. We separated labeled ribo- and deoxynucleotides on a nucleosil 100 C18 column (Phenomenex, Castel Maggiore, Italy) by isocratic elution with 0.1 M ammonium acetate, pH 7.0 (0.5-ml fractions; 0.5 ml/min), with the following retention times: GTP, 9 min; GDP, 10 min; GMP, 12 min; dGTP, 15 min; dGDP, 19 min; dGMP, 22 min; and guanine, 25 min. After 30 min we changed during a 5-min period the eluting buffer by a linear gradient from 0.1 M ammonium acetate to ammonium acetate with 30% methanol, which eluted GdR at 55 min. When we reisolated labeled GdR from cells to determine its specific radioactivity, we added 1% acetonitrile to the eluting buffer, with GdR appearing after 78 min. To obtain the specific radioactivity, we divided the total radioactivity in the peak with the amount of GdR calculated from the peak area by comparison with known standards.

The second method provided independently of the first method the size and the specific radioactivity of the dGTP pool (28, 30). To this purpose we measured the simultaneous incorporation by DNA polymerase of [3H]dGTP present in the extract with an added excess of [32P]dATP into a synthetic oligo(dT-dC) polymer. By comparison with standard curves, we could calculate the size of the dGTP pool from [32P] and the specific radioactivity from [3H] incorporated into the polymer. Incorporation of isotope into nuclear and mitochondrial DNA was determined as described earlier (20).

All of the values are given per million cells. To correct for the possibility of slight variations in the amount of cells/plate, we used the following normalization. After extraction of nucleotides from the mitochondrial fraction, we dissolved the remaining pellet (containing nuclei as well as mitochondria) in 1.5 ml of 0.3 M NaOH at room temperature overnight and after centrifugation determined the \( A_{260\,\text{nm}} \) of the supernatant. One million cells had an \( A_{260\,\text{nm}} \) of 0.65 (CHO cells) or 0.48 (human fibroblasts). These corrections rarely amounted to more than 10%.

RESULTS

General Strategy for Experiments—To determine the origin of mt deoxyguanosine phosphates and their relation to the cytosolic pools, we introduce isotope into hamster CHO cells or human fibroblasts from trace amounts of highly radioactive deoxyguanosine. The flux of isotope through mt and cytosolic guanine deoxynucleotides is a function of synthesis and consumption. In both cycling and quiescent cells the nucleoside is phosphorylated by dGK (24) in mitochondria and by dCK (25) in the cytosol (Fig. 1). We can exclude phosphorylation by dCK by carrying out experiments with CHO dCK− cells or by including nonlabeled Cdr to block dCK activity toward GdR in fibroblasts. Then the isotope first enters dGMP inside mitochondria via phosphorylation by dCK, in mitochondria via phosphorylation by dGK. Hydroxyurea (HU) blocks ribonucleotide reductase activity. The activity of the two kinases is modulated in substrate cycles by 5'-deoxynucleotidases (possibly cdN) in the cytosol and mdN in mitochondria (18). Deoxyguanosine is distributed between cytosol and mitochondria by facilitated diffusion (ENT1); guanine deoxynucleotides shuttle across the mitochondrial membrane by an unknown mechanism. Deoxynucleosine is also degraded by purine nucleoside phosphorylase (PNP), an enzyme inhibited by immucillin H (imm H). The resulting guanine is transformed to guanine ribonucleotides by HGPRT and recycled by ribonucleotide reductase to guanine deoxynucleotides.
A complication arises from the rapid degradation of GdR by purine nucleoside phosphorylase (7) followed by incorporation of the labeled guanine into the guanine ribonucleotide pool by hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Reduction of GDP to dGDP then can recycle into cytosolic guanine deoxynucleotides (Fig. 1). We can minimize recycling by inclusion of the drug immucillin H, a powerful inhibitor of deoxynucleoside degradation (31), and by using HGPRT− cells.

Activity Profiles of Homogeneous dCK and dGK—We prepared pure human recombinant dGK (24) and dCK (25) and compared the ability of each enzyme to phosphorylate GdR and CdR. In confirmation of earlier reports, we found that each enzyme phosphorylates both deoxynucleosides but that the $K_m$ values for dCK greatly favor phosphorylation of CdR, whereas the $K_m$ values for dGK favor phosphorylation of GdR. Neither enzyme strictly follows Michaelis-Menten kinetics (25). At low substrate concentrations we found for dCK a $K_m$ value of 0.2 $\mu$M for CdR and of more than 100 $\mu$M for GdR. For dGK the $K_m$ values were ~0.5 $\mu$M for GdR and 200 $\mu$M for CdR. Table 1 shows a few selected data of importance for the planning of our experiments. At equal protein concentration each enzyme phosphorylated 1 $\mu$M GdR with the same rate. An equimolar concentration of CdR decreased the phosphorylation of CdR by dCK to 3%, whereas even a 1000-fold excess of CdR barely affected the phosphorylation of 1 $\mu$M CdR by dGK. From these results we decided to use a concentration of 1 $\mu$M labeled CdR for our cell experiments and, where required, to inhibit the activity of dCK toward GdR with 5 $\mu$M nonlabeled CdR. Neither enzyme was inhibited by aphidicolin or hydroxyurea (data not shown).

Cellular Location of dCK and dGK—As a preliminary to our isotope flow experiments, we determined the activity of the two kinases in extracts from various cell lines (cycling dCK− and dCK+ CHO cells and cycling and quiescent human HGPRT− and HGPRT+ skin fibroblasts) as well as in the mitochondria and the cytosol from these cells (Table 2). We draw the following conclusions from the results: (i) dCK activity is much higher than dGK activity in both CHO cells and human fibroblasts; (ii) CdR phosphorylation by dCK occurs only in the cytosol with similar rates in cycling CHO cells and fibroblasts but with a lower rate in quiescent fibroblasts; (iii) GdR phosphorylation occurs in both the cytosol and mitochondria. The cytosolic activity is inhibited by CdR in fibroblasts and is therefore catalyzed by dCK. The inhibition is not seen in CHO cells that already contain deoxycytidine compounds. In mitochondria GdR phosphorylation is catalyzed by dGK and not inhibited by CdR. dGK activity is slightly higher in quiescent than in cycling fibroblasts, but cycling CHO cells contain by far the highest activity. Importantly, the results confirm the presence of dCK in the cytosol and dGK in mitochondria and show that the activity of the cytosolic dCK toward GdR in fibroblasts can be suppressed by CdR without affecting dGK.

Immucillin H Prevents Recycling of [3H]GdR in HGPRT− CHO Cells—The higher dGK activity of CHO cells, compared with fibroblasts (Table 2), and their rapid growth made them a preferred object for our experiments. In addition, comparing results from CHO dCK− and dCK+ cell lines allowed a clear distinction between the contributions of dCK and dGK. We therefore started our investigations with these two cell lines. In preliminary experiments CHO HGPRT− cells rapidly degraded [3H]GdR and incorporated almost 90% of the radioactivity into ribonucleotides and RNA (Fig. 1). Also in HGPRT− cells CdR was degraded with some radioactivity appearing in ribonucleotides, but the addition of immucillin H blocked this process, and the radioactivity now was present in guanine deoxynucleotides and DNA (data not shown). These results established that HGPRT− cells in the presence of immucillin H did not recycle radioactive guanine by degradation of [3H]GdR and that

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**TABLE 1**

| Substrate specificity of recombinant human deoxynucleoside kinases |
|---------------------------------------------------------------|
| The pure recombinant enzymes were assayed at different concentrations of radioactive substrates according to Refs. 24 and 25. We determined the influence of deoxycytidine on deoxyguanosine phosphorylation by increasing concentrations of nonlabeled deoxycytidine to the reaction mix containing 1 $\mu$M [3H]deoxyguanosine. ND, not determined. |

| Substrate concentration | Enzyme activity | dCK | dGK |
|-------------------------|----------------|-----|-----|
| Deoxycytidine           | Deoxyguanosine | $\mu$M | umol nucleotide/mg enzyme/min |
| 1                       | 0              | 55a | 0.38a |
| 10                      | 0              | 65a | 3.1a  |
| 0.1                     | 1              | 27a | 3.3a  |
| 0.1                     | 1              | 16a | ND    |
| 10                      | 1              | 0.82a | ND |
| 1000                    | 1              | 0b  | 27b   |

* Deoxycytidine as substrate.
* Deoxyguanosine as substrate.

**TABLE 2**

| Kinase activities in extracts and subcellular fractions from CHO cells and human fibroblasts |
|------------------------------------------------------------------------------------------|
| We measured the phosphorylation of [3H]GdR and [3H]CdR in extracts from cells, mitochondria, and the cytosol of the different cell lines as described under “Experimental Procedures.” The reported results show the ranges of variation in separate extracts with the numbers of extracts in parentheses. 1 microunit = 1 pmol of deoxynucleoside monophosphate formed per min. |

| Substrate | Enzyme activity | CHO Cells | Mitochondria |
|-----------|----------------|-----------|--------------|
| GdR       |                 | Whole cell | Cytosol      |
| CdR       |                 | Whole cell | Cytosol      |
| [3H]CdR   |                 | Whole cell | Cytosol      |

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16440
labeling of deoxynucleotides and DNA occurred exclusively by direct phosphorylation of GdR, catalyzed either by dGK or dCK.

[^3]H]GdR Is Transported into Cells with Little Dilution of Isotope—The transport of[^3]H]GdR from the medium across the cell membrane depends mainly on concentrative nucleoside transporters (32). For the interpretation of the results from the isotope experiments described below, we wished to know whether the specific radioactivity of the imported labeled GdR was diluted by nonlabeled intracellular GdR. We incubated 40 million CHO HGPRT− dCK− cells with 1 μM[^3]H]GdR (290 cpm/pmol) and immucillin H, purified after 20 min[^3]H]GdR from the cytosol by HPLC and determined its specific radioactivity as described under “Experimental Procedures.” In three separate determinations we found values of 263, 303, and 433 cpm/pmol, suggesting little or no dilution of the externally added[^3]H]GdR by endogenous nonlabeled GdR.

Effect of Extracellular[^3]H]GdR Concentration on Intracellular Isotope Content—We determined how increasing the concentration of[^3]H]GdR in the medium from 0.2 to 1.0 μM affected the phosphorylation of the nucleoside by CHO HGPRT− dCK− cells. After 20- and 60-min incubations, we determined in the cytosol and in mitochondria the total radioactivity in GdR, guanine deoxynucleotides (dGMP + dGDP + dGTP), and DNA (Fig. 2). We record only the combined total radioactivity of the three deoxynucleotides because the distribution of radioactivity between dGMP, dGDP, and dGTP did not vary in different experiments, with dGTP accounting for 65–75% in the cytosol and 30–50% in mitochondria.

The increase of the extracellular concentration of GdR from 0.2 to 1.0 μM gave a corresponding 5-fold increase in the radioactivity of intracellular GdR in the cytosol and a 2.5-fold increase in mitochondria (Fig. 2). As expected from the low K_m value of dGK for GdR, the phosphorylation of the nucleoside was only marginally increased: 1.5-fold in the cytosol and 1.2-fold in mitochondria. The size of the two dGTP pools did not increase, and in consequence their specific radioactivities increased in parallel to their total radioactivity (Fig. 2). In further experiments we employed an extracellular concentration of 1 μM[^3]H]GdR to label the nucleotide pools of cells. This concentration provided sufficient radioactivity to follow the flux of isotope in cells without unduly disturbing their metabolism.

Isotope Flux in Cytosolic and mt Guanine Deoxynucleotides of CHO Cells—We next incubated cycling cultures of dCK− or dCK+ cells for up to 80 min with labeled GdR and carried out a chase after 20 min by replacing the isotopic medium of some cultures with nonlabeled medium, either with or without 1 μM cold deoxyguanosine to determine the decay of radioactivity during the ensuing 60 min. We determined both the total radioactivity in deoxynucleotides and the specific radioactivity of dGTP in the cytosolic and mt pools. The experiment with dCK− cells is depicted in Fig. 3 (A and B), with Fig. 3A showing the total radioactivity of the three deoxynucleotides and DNA and Fig. 3B showing the specific radioactivity of dGTP. Fig. 3 (C and D) represents the corresponding experiment with dCK+ cells.

The time curves in Fig. 3 (A and C) represent the accumulated phosphorylation of GdR. In dCK− cells (Fig. 3A) all phosphorylation occurred in mitochondria. The rapid appearance of radioactivity in cytosolic deoxynucleotides demonstrates their export from mitochondria. After 20 min the total radioactivity of both the cytosolic and mt pools reached equilibrium with similar plateaus. Incorporation into DNA, instead, continued linearly during the whole time period with almost 90% of the radioactivity being in DNA after 80 min (note the difference in scales between DNA and guanine deoxynucleotides in Fig. 3, A and C). During the chase period both the mt and cytosolic pools rapidly lost radioactivity, with the cytosolic pool decaying faster than the mt pool. The presence of nonlabeled deoxyguanosine during the chase did not affect the results (not shown). The labeling patterns suggest a dynamic equilibrium between the mt and cytosolic pools with continuous synthesis of mt deoxyribonucleotides from GdR, followed by transfer of guanine...
deoxynucleotides to the cytosolic pool and subsequent incorporation into DNA.

We obtained similar results with dCK+ cells (Fig. 3C). The total radioactivity in both nucleotide pools and DNA was, however, almost two times larger in dCK+ cells, and a complete equilibrium between the pools had not yet been established after 20 min, suggesting that continued phosphorylation of GdR by dCK increased the radioactivity of the cytosolic pool. Also in these cells the radioactivity of the nucleotide pools decayed rapidly during the chase period.

Fig. 3 (B and D) shows the specific radioactivity of dGTP. We can only measure the specific radioactivity of dGTP but assume that dGMP and dGDP had closely similar values. The specific radioactivity is obtained by division of the radioactivity in the dGTP pool with its size (here 0.32 pmol/million cells in mitochondria and 4 pmol in the cytosol). In our experiments the dGTP pool was replenished continuously by phosphorylation of nonlabeled GDP and the specific radioactivity of dGTP was a function of the relative rates of the two processes. In dCK+ cells the highest specific radioactivity of dGTP was 10% of that of the precursor GdR, demonstrating also here a dilution by unlabeled guanine deoxynucleotides that must have entered from the cytosol. During the chase period the specific radioactivity of both the cytosolic and the mt dGTP pool decreased rapidly (Fig. 3, B and D), demonstrating a fast turnover of the mt dGTP pool.

Inhibition by Aphidicolin or Hydroxyurea—Aphidicolin stops nuclear DNA replication (33) and thus reduces the requirements of the cell for dNTPs. We determined the effect of aphidicolin on the flux of isotope through guanine deoxynucleotides in an experiment similar to that shown in Fig. 3 for dCK+ cells that served as control. The drug severely inhibited the incorporation of isotope into DNA but affected deoxynucleotide labeling only marginally, with no change for the mt pool and a minor increase in the labeling of the cytosolic pool (compare Fig. 3A and Fig. 4A). Both pools reached equilibrium already after 10 min and lost radioactivity during the chase period. We used shorter time intervals for the chase than in the corresponding control experiment shown in Fig. 3 to investigate whether the drug might affect the turnover of dGTP early during the chase. This was, however, not the case. Aphidicolin did not change the specific radioactivity of the dGTP pools (compare Figs. 3B and 4B) or their size.

From enzyme experiments we know that the drug does not inhibit the phosphorylation of GdR by dGK. The data in Fig. 4 now suggest that aphidicolin did not materially inhibit ribonucleotide reduction or the flux of guanine deoxynucleotides between mitochondria and the cytosol, nor did it affect the loss of isotope during the chase. We propose that in the absence of dGTP incorporation into DNA the cells degraded dGMP by
substrate cycles (19) and that excretion of deoxyguanosine into
the medium substituted for incorporation of dGTP into DNA.
In this way a dynamic equilibrium similar to that in Fig. 3 was
maintained.

Hydroxyurea had a more profound effect in dCK− cells. The
drug inhibits primarily the de novo synthesis of deoxyribo-
nucleotides by blocking ribonucleotide reduction (34) and
thereby also inhibits DNA replication. In a preliminary exper-
iment we found that preincubation for either 30 or 60 min
with hydroxyurea led to a large accumulation of radioactivity in
cytosolic guanine deoxynucleotides irrespective of the time of
preincubation (data not shown). In a second more extensive
experiment (Fig. 4, C and D) we extended the time of incubation
with [3H]GdR to 120 min and included after 60 min a 60-min
chase with nonlabeled medium. The results from a parallel con-
throl experiment without hydroxyurea were in all respects simi-
lar to those in Fig. 3 and are not shown.

Hydroxyurea greatly increased the radioactivity of cytosolic
guanine deoxynucleotides (Fig. 4C, note the difference in scale
from Fig. 3A), with a smaller increase in the mt pool. Already
after 60 min the cytosolic pool had increased 8-fold, and a simi-
lar large increase occurred in dGTP specific radioactivity
(compare Figs. 4D and 3B). The size of the dGTP pools did not
change significantly (data not shown). In the absence of the
drug, the total radioactivity in guanine deoxynucleotides
reached a plateau after 20 min (Fig. 3A), whereas with
hydroxyurea it increased continuously during the whole 120-
min incubation period (Fig. 4C).

The accumulation of radioactivity did not depend on
increased phosphorylation of radioactive deoxyguanosine but
was caused by decreased degradation as shown from the results
of the chase. Fig. 5 compares the decrease in the specific radio-
activities of cytosolic and mt dGTP from the hydroxyurea
experiment with similar data from the earlier experiments
without hydroxyurea. The curves show the percentage of
decrease with time with the values before the chase set to 100.

In the presence of hydroxyurea, the specific radioactivity
of cytosolic dGTP (Fig. 5A) decreased with a half-life of ~40 min,
whereas in the controls and in the presence of aphidicolin the
half-lives were only a few minutes. This strikingly decreased
turnover of the pool was probably because of inhibition of ribo-
nucleotide reduction, not because of inhibition of DNA synthe-
sis. The result differs from the effect of aphidicolin where the
inhibition of DNA synthesis only marginally decreased the
turnover, as shown from the decay during the chase (Fig. 5A).
The turnover of the mt dGTP pool was much less affected by
hydroxyurea (Fig. 5B).

Hydroxyurea decreased the incorporation of labeled dGTP
into DNA but did not inhibit it completely (Fig. 4C). The incor-
poration was nonlinear and decreased with time, indicating a
gradual inhibition of DNA synthesis. The nonlinearity with
time of the increase in DNA labeling and in the specific radio-
activity of dGTP exclude a precise quantitation, but we estimate
that between 60 and 120 min DNA synthesis was decreased to
~1%.

Isotope Flux in Cycling and Quiescent Human Fibroblasts—
We were not able to make CHO cells quiescent, and all our
experiments therefore had to be done with cycling cells. To
compare cycling and quiescent cells we turned to a human
HGPRT− dCK− fibroblast line. These cells lack an active
HGPRT and do not recycle guanine from labeled deoxy-
guanosine. Their dCK activity can be suppressed with CdR in
the medium to distinguish dGK and dCK activities. After
reaching confluence the fibroblasts could be maintained in
the quiescent state in 0.1% fetal calf serum. Before conflu-
ence they grew sluggishly, with a generation time of 2–3 days
and with only 10–15% of the cells in S phase, as compared
with 50–70% in cycling CHO cells. Cell extracts from cycling
fibroblasts had the same dCK activity as CHO extracts, but
their dGK activity was much lower, in quiescent fibroblasts
dGK activity was slightly higher than in cycling cells, and
dCK decreased (Table 2).

We show here the results from one of several experiments
where we labeled cycling and quiescent cells with [3H]GdR in
the presence or absence of CdR (Fig. 6). With CdR the phos-
phorylation of GdR by dCK is suppressed, and radioactivity
is introduced into guanine deoxynucleotides exclusively by
the mt dGK. Similar to CHO cells (Fig. 3), the flux of isotope
through cytosolic and mt deoxynucleotide pools of cycling
fibroblasts (Fig. 6, A, C, and E) reached equilibrium after 20
min. There are, however, quantitative differences. With CdR
in the medium, mitochondria from cycling fibroblasts (Fig.
6C) contained only one-sixth of the radioactivity of CHO
dCK− cells (Fig. 3A), probably reflecting the higher dGK
activity of the latter. In the cytosol (Fig. 6A) there was only a
minor difference between fibroblasts and dCK− CHO cells
(Fig. 3A). When the cells were incubated without CdR and
dCK therefore contributed to GdR phosphorylation the
radioactivity of the cytosolic deoxynucleotides increased
about 4-fold, suggesting that 70–80% of GdR phosphoryla-
tion in cells normally was provided by dCK. The 5-fold lower
specific activity of the cytosolic dGTP (Fig. 6E) in the pres-
ence of CdR confirms this conclusion.

The cytosolic dGTP pool of quiescent fibroblasts (2.9 pmol/
mill cells) was only half the size of cycling fibroblasts (5.8 pmol/
mill cells). Nevertheless the kinetic behavior of the two pools
was similar, and both reached isotope equilibrium after 20 min
(Fig. 6, B, D, and F). However, blocking dCK activity with
deoxycytidine affected quiescent cells much less than cycling
cells, indicating a smaller contribution of dCK, in accordance
with the lower activity of the enzyme in extracts from quiescent

The turnover of the pool was probably because of inhibition of ribo-
nucleotide reduction, not because of inhibition of DNA synthe-
sis. The result differs from the effect of aphidicolin where the

Guanine Deoxyribonucleotides and Mitochondrial DNA

FIGURE 5. Effects of hydroxyurea and aphidicolin on the turnover of
dGTP. We show the time-dependent percentage of decrease of the specific
radioactivity of dGTP during the chase of the experiments in Figs. 3 and 4 with
the starting values set at 100. A, cytosolic dGTP; B, mt dGTP. ○, control (dCK−);
●, control (dCK−); □, hydroxyurea; ▲, aphidicolin.
We find a close metabolic connection between mt and cytosolic dNTP pools. They are synthesized and maintained by a common network of enzymes distributed between cytosol and mitochondria, as shown in Fig. 1 for dGTP, and communicate rapidly with each other. For a long time the prevailing view was that specific kinases inside mitochondria form mt dNTPs, whereas de novo synthesis by ribonucleotide reductase provides cytosolic dNTPs, with the two pools separated by the mt membrane. Autoradiographic observations (35, 36) had shown specific labeling of mt DNA from deoxynucleosides, and metabolic experiments claimed depletion of cytosolic but not of mt dTTP after inhibition with amethopterin (37). Support also came from the discovery that in humans genetic deletion of intra-mt deoxynucleoside kinase activity results in depletion of mt DNA (4, 5).

By determining the flux of labeled nucleotides in intact cells, we find instead a two-way communication between mt and cytosolic dNTPs. In earlier experiments (20–23) radioactivity introduced specifically into mt dTTP from $[^3H]$thymidine was rapidly redistributed to cytosolic dTTP. The labeled pools reached within minutes a steady state during which phosphorylation of thymidine and consumption of dTTP were in balance.

We now use deoxyguanosine to label guanine deoxyribotides and determine their flux in cells under normal conditions and after inhibition of DNA replication with aphidicolin or inhibition of ribonucleotide reduction with hydroxyurea. The results lead to the following conclusions: (i) Cytosolic and mt guanine deoxynucleotides are in a dynamic equilibrium and rapidly attain an isotopic steady state in both cycling and quiescent cells. They are continuously synthesized by dGK in mitochondria and by dCK and ribonucleotide reductase in the cytosol. They are consumed for DNA replication and by substrate cycles. (ii) mt guanine deoxynucleotides are exported from mitochondria to the cytosol. In cells lacking dCK activity $[^3H]$deoxyguanosine is incorporated into deoxynucleotides exclusively in mitochondria by dGK and rapidly transported to the cytosol. In these cells the specific radioactivity of dGTP is at equilibrium much higher in mitochondria, suggesting that radioactive dGTP in the cytosol is diluted by nonradioactive deoxynucleotides formed by the cytosolic ribonucleotide reductase. (iii) Guanine deoxynucleotides enter mitochondria from the cytosol. The specific radioactivity of mt deoxynucleotides never reaches the specific radioactivity of deoxyguanosine but stabilizes at equilibrium at a value of between 10 and 20%, which suggests an influx of dGTP with low specific radioactivity from the cytosol. (iv) Ribonucleotide reduction in the cytosol provides the bulk of guanine deoxynucleotides in both cycling and resting cells. This appears from the low specific radioactivity of cytosolic dGTP at isotope equilibrium showing a high dilution of radioactivity by nonlabeled dGTP. (v) Both mt and cytosolic deoxynucleotides rapidly lose isotope in chase experiments demonstrating a high pool turnover, also in resting cells and when DNA synthesis is inhibited with aphidicolin in cycling cells. This suggests that in the absence of DNA synthesis ribonucleotide reduction continues and that the size of the dGTP pool is maintained by increased degradation of dGMP with excretion of deoxyguanosine into the mitochondria, whereas specific kinases inside mitochondria form mt dNTPs, and communicate rapidly with each other. For a long time the prevailing view was that specific kinases inside mitochondria form mt dNTPs, whereas de novo synthesis by ribonucleotide reductase provides cytosolic dNTPs, with the two pools separated by the mt membrane. Autoradiographic observations (35, 36) had shown specific labeling of mt DNA from deoxynucleosides, and metabolic experiments claimed depletion of cytosolic but not of mt dTTP after inhibition with amethopterin (37). Support also came from the discovery that in humans genetic deletion of intra-mt deoxynucleoside kinase activity results in depletion of mt DNA (4, 5).

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**FIGURE 6.** Isotope flux in cytosolic and mitochondrial guanine deoxynucleotide pools of cycling and quiescent human fibroblasts. We incubated separate cultures of cycling (A, C, and E) or quiescent (B, D, and F) human HGPRT$^+$ skin fibroblasts with 1 μM $[^3H]$GdR and 0.5 μM immucillin H with a chase after 20 min, as described for Fig. 3. In each experiment half of the cultures contained 5 μM CDR (filled symbols) to block the phosphorylation of $[^3H]$GdR by dCK, or no CDR (open symbols). A and B, total radioactivity in cytosolic guanine deoxynucleotides. C and D, total radioactivity in mt guanine deoxynucleotides. E and F, specific radioactivity of cytosolic dGTP. □ and ■, cytosolic deoxynucleotides or dGTP; ◦ and ○, mt deoxynucleotides.

cells (Table 2). The chase experiments suggest a rapid pool turnover in both cycling and quiescent cells, slower in mitochondria than in the cytosol. We observed that quiescent cells incorporated hardly any dGTP into DNA; therefore the turnover indicates degradation of the pools by substrate cycles and excretion of GdR into the medium.

In general terms the metabolism of guanine deoxynucleotides in cycling human fibroblasts closely resembles the metabolism in cycling hamster CHO cells, and also quiescent fibroblasts behave in a surprisingly similar manner. Observed differences in details in labeling patterns can be explained from variations in enzyme content and growth rate.

**DISCUSSION**

Measurements of enzyme activities and pool sizes are snapshots of cells. They tell us little about the dynamic aspects of cell metabolism. The size of a dNTP pool depends on both its synthesis and its consumption. The isotope flux methodology employed by us here and earlier measures both synthesis and turnover of pools and above all permits us to draw conclusions about interrelations within the anabolic and catabolic enzymatic network underlying these processes.
enzymes of the network. The depletion of any of the involved dNTPs is provided only by the combined actions of all anabolic activity alone does not suffice. An alternative explanation might be that the required appropriate balance between all four ciency targeting liver and brain. In these cases apparently genetic deficiencies of mt kinases also result in mt DNA deple-

during the cycling state in agreement with the prevalence of the enzyme.

A comparison between the metabolic flux in cycling and quasi-
sence cells indicates only small differences. In both cases a dynamic equilibrium is rapidly established and maintained as described above. One apparent difference concerns the contribu-
tion of dCK to the phosphorylation of GdR, which is larger during the cycling state in agreement with the prevalence of the enzyme.

In the cells investigated here ribonucleotide reductase is of overiding importance for the supply of dGTP. In quiescent cells p53R2 forms part of the active enzyme, and indeed it was recently found that its genetic deficiency in humans results in a severe depletion of mt DNA with early death of the affected individuals (6) demonstrating that the mt kinases (Fig. 1) did not provide sufficient dNTPs for a backup, because of either insufficient activity or substrate availability. On the other hand genetic deficiencies of mt kinases also result in mt DNA deple-
tion, TK2 deficiency targeting skeletal muscle, and dGK defi-
ciency targeting liver and brain. In these cases apparently p53R2 activity cannot provide a dNTP normally produced by the missing kinase. Possibly in the affected organs the reductase activity alone does not suffice. An alternative explanation might be that the required appropriate balance between all four dNTPs is provided only by the combined actions of all anabolic enzymes of the network. The depletion of any of the involved enzymes might then disturb the balance.

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JUNE 13, 2008 • VOLUME 283 • NUMBER 24

JOURNAL OF BIOLOGICAL CHEMISTRY 16445