

# Mitochondrial Deoxynucleotide Pools in Quiescent Fibroblasts

A POSSIBLE MODEL FOR MITOCHONDRIAL NEUROGASTROINTESTINAL ENCEPHALOMYOPATHY (MNGIE)\*

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Mitochondrial (mt) DNA depletion syndromes can arise from genetic deficiencies for enzymes of dNTP metabolism, operating either inside or outside mitochondria. MNGIE is caused by the deficiency of cytosolic thymidine phosphorylase that degrades thymidine and deoxyuridine. The extracellular fluid of the patients contains 10–20  $\mu\text{M}$  deoxynucleosides leading to changes in dTTP that may disturb mtDNA replication. In earlier work, we suggested that mt dTTP originates from two distinct pathways: (i) the reduction of ribonucleotides in the cytosol (in cycling cells) and (ii) intra-mt salvage of thymidine (in quiescent cells). In MNGIE and most other mtDNA depletion syndromes, quiescent cells are affected. Here, we demonstrate in quiescent fibroblasts (i) the existence of small mt dNTP pools, each usually 3–4% of the corresponding cytosolic pool; (ii) the rapid metabolic equilibrium between mt and cytosolic pools; and (iii) the intra-mt synthesis and rapid turnover of dTTP in the absence of DNA replication. Between 0.1 and 10  $\mu\text{M}$  extracellular thymidine, intracellular thymidine rapidly approaches the extracellular concentration. We mimic the conditions of MNGIE by maintaining quiescent fibroblasts in 10–40  $\mu\text{M}$  thymidine and/or deoxyuridine. Despite a large increase in intracellular thymidine concentration, cytosolic and mt dTTP increase at most 4-fold, maintaining their concentration for 41 days. Other dNTPs are marginally affected. Deoxyuridine does not increase the normal dNTP pools but gives rise to a small dUTP and a large dUMP pool, both turning over rapidly. We discuss these results in relation to MNGIE.

A diploid animal cell contains two copies of nuclear DNA and hundreds to thousands of copies of mitochondrial (mt)<sup>1</sup> DNA. Nuclear DNA replication occurs only during S-phase, once dur-

ing the life cycle of a cell. It is strictly regulated and limited to growing cells. Little is known regarding the regulation of mtDNA, but its replication occurs also outside S-phase (1). All of the DNA replication requires pools of the four dNTPs. The mt pools are localized in the mt matrix, separated by the impermeable mt inner membrane from the cytosolic pools that serve for nuclear DNA replication. In recent years, several genetic diseases have been described that affect mtDNA replication (2, 3). In some instances, the cause for the disease lies in the malfunction of enzymes directly involved in the replication of mtDNA, but in other instances, the cause appears to be an enzyme involved in the metabolism of dNTPs. A better understanding of these diseases requires an understanding of the synthesis and metabolism of mt dNTPs under normal and pathological conditions.

Mitochondrial DNA has been reported to amount to ~5% nuclear DNA (4). Also, the mt dNTP pool represents only a fraction of the total dNTP pool (5). We recently described a reliable methodology for the quantitative separation of mt and cytosolic dNTP pools that made it possible to determine their size and to study their metabolism by isotope experiments with [<sup>3</sup>H]thymidine (6). Our results with cycling tumor cells suggested the existence of two independent pathways for mt dNTP synthesis as depicted in the model for the synthesis of mt dTTP shown in Fig. 1. In contradiction to a common belief, we found that thymidine phosphates were rapidly exchanged between the cytosol and mitochondria. In growing cells, the major source for mt dTTP is the reduction of ribonucleotides followed by methylation of the resulting dUMP in the cytosol (Fig. 1) (7). A transporter located in the mt membrane then transports the deoxynucleotide (probably as the diphosphate) into the mt matrix (8) where it serves for the synthesis of mtDNA. It seems likely that corresponding mechanisms also transfer the other three cytosolic deoxynucleotides into the mt matrix. The second pathway becomes important in quiescent cells. Here ribonucleotide reductase is strongly reduced or absent and insufficient deoxynucleotides are synthesized *de novo*. Instead, the cells transport thymidine from the cytosol into mitochondria (Fig. 1) and phosphorylate the nucleoside with a mitochondrial thymidine kinase (TK2) (9, 10). The cytosolic thymidine originates from the extracellular fluid and from intracellular degradation. A separate thymidine kinase (TK1) salvages thymidine in the cytosol (11). Both in the cytosol and in mitochondria, 5'-deoxynucleotidases (cdN in the cytosol and mdN in mitochondria) oppose the reaction catalyzed by the two thymidine kinases by dephosphorylating dTMP to thymidine (12). In each compartment, one kinase and one deoxynucleotidase form a substrate (= futile) cycle that regulates dTMP synthesis and, consequently, also the size of the dTTP pool (see Fig. 1) (5, 13). In the cytosol, one more enzyme, thymidine phosphorylase (14), interlocks with the substrate cycle. This enzyme degrades thymi-

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<sup>1</sup> The abbreviations used are: mt, mitochondrial; TLC, thin layer chromatography; MNGIE, mitochondrial neurogastrointestinal encephalomyopathy; TK1, cytosolic thymidine kinase; TK2, mitochondrial thymidine kinase; dGK, mitochondrial deoxyguanosine kinase; cdN, cytosolic 5'-deoxynucleotidase (formerly dNT-1); mdN, mitochondrial 5'-deoxynucleotidase (formerly dNT-2); FCS, fetal calf serum; BVdU, bromovinyldeoxyuridine; DPB-T, (S)-1-[2'-deoxy-3',5'-O-(1-phosphono)-benzylidene- $\beta$ -D-threo-pentofuranosyl]thymine; PMcP-U, ( $\pm$ )-1-trans-(2-phosphonomethoxycyclopentyl)-uracil; HPLC, high pressure liquid chromatography.

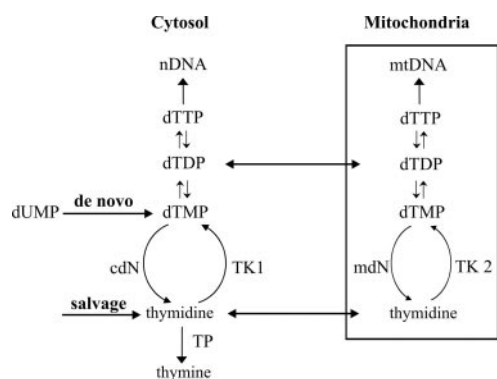


FIG. 1. **Relations between cytosolic and mitochondrial thymidine phosphates.** In the cytosol, thymidine phosphates are synthesized (i) by *de novo* reduction of ribonucleotides followed by methylation of dUMP or (ii) by salvage through phosphorylation of thymidine by a cytosolic thymidine kinase (TK1). The salvage pathway is kept in check at the level of dTMP by a substrate cycle between TK1 and cdN and at the level of thymidine by thymidine phosphorylase (TP). In mitochondria, a separate mitochondrial thymidine kinase (TK2) phosphorylates thymidine. TK2 forms a second substrate cycle with mdN. Thymidine phosphates are rapidly exchanged between mitochondria and cytosol.

dine to thymine and thereby removes one component of the cycle directing its activity in the catabolic direction. This system provides the cell with an intricate mechanism to regulate the dTTP pool. It appears to be an important safeguard against overproduction of dTTP. This view is strengthened by the recent discovery that genetic deficiency of thymidine phosphorylase leads to MNGIE (15). In the absence of this enzyme, the degradation of thymidine is largely abolished. Body fluids of the afflicted individuals contain large amounts (10–20  $\mu\text{M}$ ) of thymidine and deoxyuridine (16, 17), probably resulting in an increase of the intracellular dTTP pool and possibly also derangements of other dNTP pools. However, it is not clear why derangements in the cytosol would give rise to a mt disease.

MNGIE attests to the deleterious effects of too much dTTP. However, also insufficient dTTP can result in disease. The genetic loss of TK2 activity causes a mtDNA depletion syndrome with isolated skeletal myopathy (18). In the absence of TK2, the second pathway of our model apparently does not produce mt dTTP. Because this pathway only should be required in quiescent cells, we would expect that only in such cells the absence of TK2 would result in a deficiency of mt dTTP.

Are the two pathways in Fig. 1 valid for dTTP only or also for other mt dNTPs? Mitochondria contain, in addition to TK2, a deoxyguanosine kinase (dGK) (19). Also, the absence of this enzyme activity leads to a mtDNA depletion syndrome (20). Experiments with cultured patient fibroblasts suggest that mtDNA is compromised in quiescent cells but only mildly affected during exponential growth (21). Therefore, we hypothesize the general rule that mt dNTPs derive from two pathways: 1) active in growing cells, relying on the import of deoxynucleotides synthesized *de novo* in the cytosol, and 2) active in quiescent cells, relying on the import of deoxynucleosides and their phosphorylation by intramitochondrial kinases.

The present work has two major aims: (i) to study the relation between mt and cytosolic dNTP pools in contact-inhibited fibroblasts as representatives of quiescent cells (earlier results (5, 6) were from tumor cells in exponential growth), and (ii) to provide a model for MNGIE by investigating changes induced in quiescent fibroblasts by thymidine and/or deoxyuridine. Our results further support the model of Fig. 1 and suggest possible mechanisms for the etiology of MNGIE.

## EXPERIMENTAL PROCEDURES

**Materials**— $[^3\text{H}]$ Thymidine (20,000 cpm/pmol) was from PerkinElmer Life Sciences.  $[5\text{-}^3\text{H}]$ Deoxyuridine (20,000 cpm/pmol) was from Moravick (Brea, CA). The first batch of the nucleoside was labeled exclusively in the 5-position of uracil, but subsequent batches were all contaminated with some isotope in the 6-position. Therefore, after incubation with cells, we found considerable amounts of radioactivity in thymidine phosphates and DNA. It was not possible to get a clean batch again, which complicated the analyses of our experiments greatly. The inhibitors of 5'-deoxynucleotidases, DPB-T and PMcP-U (22), were from Dr. Jan Balzarini, (Rega Institute, Katholieke Universiteit, Leuven, Belgium). BVdU was from Sigma. dUTPase was a gift from Dr. R. Persson (Lund University).

**Cell Lines and Cell Growth**—Dr. Roberta Tiozzo (University of Modena) donated human skin fibroblasts. Three different lines from separate healthy donors were used between the 4th and 20th passage. An established line of lung fibroblasts (CCD 34Lu) was from the American Type Culture Collection. New cell cultures were started at a density of 0.4–0.5 million/10-cm dish and were routinely grown either on 10-cm dishes or in 75-cm<sup>2</sup> tissue culture flasks in DMEM and 10% fetal calf serum (FCS) + non-essential amino acids + 20 mM Hepes buffer, pH 7.4, at 37 °C in a humidified incubator and 5% CO<sub>2</sub>. Fresh medium was supplied twice per week.

To obtain quiescent cells, both skin and lung fibroblasts were first grown to contact inhibition within 7–9 days at which point the concentration of FCS was decreased to 0.1%. They were maintained in these conditions for 1–2 weeks with two changes of medium per week and then used for the experiments with quiescent cells. Before the change to 0.1% FCS, a 10-cm dish of skin fibroblasts contained ~4 million cells and a dish of lung fibroblasts contained 10–15 million cells. After 1–2 weeks in 0.1% serum, <1% skin fibroblasts were in S-phase as judged by fluorescence-activated cell sorter analysis and had lost all TK1 activity (an indicator of S-phase cells, see Table I). Under similar circumstances, lung fibroblasts retained a percentage of their original TK1 activity and fluorescence-activated cell sorter analyses demonstrated the presence of 2–3% S-phase cells. Lung cells required several weeks in 0.1% serum before all TK1 activity had disappeared. However, experimental results changed little after 1 week at confluence and, in most cases, we used a window of 1–2 weeks for quiescent lung cells. Maintenance in 0.1% serum resulted in detachment and loss of cells during medium change, and in most experiments, approximately half of the cells had been lost during maintenance in 0.1% serum by the time we conducted the experiment.

When we determined the effect of concentrations between 10 and 40  $\mu\text{M}$  thymidine and/or deoxyuridine on quiescent cells, we continued incubation in 0.1% FCS in the presence of one or both deoxynucleosides. HPLC analyses of the medium indicated that both deoxynucleosides were rapidly degraded with the appearance of thymine and uracil in the medium. Therefore, it was necessary to continuously add deoxynucleosides during the course of the experiments. For practical reasons, we chose the following procedure. At day 0, the deoxynucleoside(s) was added to a final concentration of 40  $\mu\text{M}$ . At day 3, the deoxynucleoside(s) was added to a final concentration of 35  $\mu\text{M}$ . At day 5, the deoxynucleoside(s) was added to a final concentration of 40  $\mu\text{M}$  + fresh medium. As a result, the concentration of a deoxynucleoside never dropped below 10  $\mu\text{M}$ .

**Isotope Experiments**—2 h before the start of an experiment, we removed the dishes from the incubator and moved them to a 37 °C thermostatic room. All of the manipulations outside the incubator were from here on done in this room. We substituted the old medium with 4 ml of fresh medium containing the desired concentration of dialyzed FCS. After 2 h, we added the isotopic deoxynucleoside and incubated the cells for the desired time period. In chase experiments, we replaced the labeled medium with fresh medium containing the non-labeled nucleoside at the original concentration. Before addition to the cells, the chase medium had been kept in the 37 °C incubator overnight. After the final incubation, we put the dishes on ice and moved them to a cold room where we made all of the further manipulations. We poured off the medium, washed the cells four times with 7–8 ml of phosphate-buffered saline, and drained them carefully. To determine dNTPs in the cytosol, we extracted whole cells on each dish with 2 ml of 60% methanol at –20 °C for 1 h, removed the methanol, and treated the solution in a boiling water bath for 3 min with care taken not to lose any methanol. We evaporated the solvent in a Speedvac centrifuge, dissolved the dry residue in a small amount of water and used it for further analyses. We dissolved the cells after methanol extraction in 2 ml of 0.3 M NaOH and used the lysate for DNA analyses. In experiments concerning dNTPs in

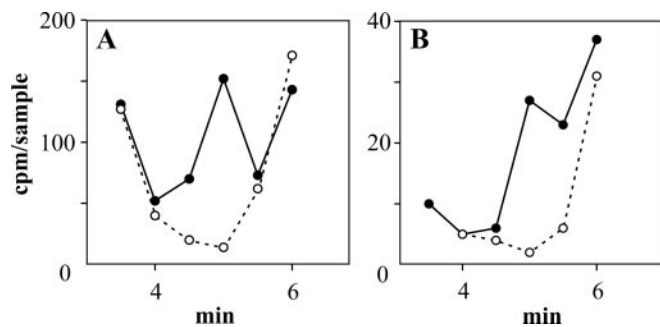


FIG. 2. **Determination of dUTP by HPLC.** Nucleotides from portions of a cytosolic (panel A) and a mt (panel B) extract from lung fibroblasts incubated with [ $^3\text{H}$ ]deoxyuridine were separated by HPLC before and after treatment with dUTPase, and the radioactivity of the chromatographic fractions was determined. The peak at 5 min is at the position of dUTP. The difference in radioactivity of this peak before and after dUTPase treatment was taken as dUTP. ●, before dUTPase; ○, after dUTPase.

mitochondria, we scraped the cells from the dish, homogenized them, and separated a combined mt and nuclear fraction from the cytosol (6). We extracted the mt fraction with 60% methanol as described above. The residue after methanol extraction served for DNA analyses

**Analytical Procedures: Enzymes**—We determined the activities of TK1, TK2, cdN, and mdN in crude cell extracts by established procedures. We used the specific inhibition of TK2 by BVdU (23) to distinguish between the two thymidine kinases. Inclusion of 50  $\mu\text{M}$  BVdU with 1  $\mu\text{M}$  thymidine as substrate did not affect TK1 but inhibited TK2 by >90%. Similarly, we could distinguish cdN from mdN from the inhibition of the latter enzyme by DBP-T (22, 24). One enzyme unit is defined as the formation of 1  $\mu\text{mol}$  of product/min. Specific enzyme activity is 1 milliunit/mg protein.

**HPLC of Nucleotides**—We separated radioactive thymidine phosphates from each other and from nucleosides by either HPLC or thin layer chromatography (TLC). In the former case, we used a C-18 column (Phenomenex, Torrance, CA) with isocratic elution (1 ml/min) with 0.1 M ammonium phosphate, pH 3.5, for 25 min followed for 35 min by a linear gradient between 0.1 M ammonium phosphate and 0.1 M ammonium phosphate in 10% methanol. Retention times (min) were as follows: dUTP, 4.8; dTTP, 7.9; dUMP, 9.8; dTDP, 10.0; uracil, 11.2; thymine, 18.6 dTMP, 19.8; and thymidine: 50.1. We collected 0.5-ml fractions and determined their radioactivity by scintillation counting.

**TLC of Nucleotides**—We used washed DC Plastikfolien PEI-cellulose (Merck). We first developed the chromatogram with water up to half of the length of the sheet to move out nucleosides from nucleotides, dried the sheet in air, and in a second step separated the nucleotides with 0.5 M ammonium formate, pH 3.6. We localized the different compounds from the position of internal carriers, cut out the corresponding parts of the sheet, placed them in scintillation vials, extracted them with 2 ml of 1 M HCl for 30 min on a shaker and determined their radioactivity after the addition of 15 ml of scintillation fluid. The recovery of radioactivity of dTTP was 80%.

**Determination of dUTP**—Only minute amounts of dUTP were recovered after incubation of cells with labeled deoxyuridine. To verify the identity of the nucleotide, we treated each extract with dUTPase and then chromatographed it to ascertain that the radioactivity in the presumed dUTP peak had disappeared. We incubated a portion of the extract in a final volume of 0.02–0.05 ml with 0.005 mg/ml UTPase and 5 mM dithiothreitol for 30 min at 30  $^{\circ}\text{C}$ , stopped the reaction by boiling, and separated labeled nucleotides either by TLC or HPLC. We could use TLC in the experiment shown in Fig. 9A when the labeled deoxyuridine contained no  $^3\text{H}$  in the 6-position of uracil. In latter experiments, all of the batches of deoxyuridine were contaminated with 6-labeled deoxyuridine resulting in the labeling of thymidine phosphates and we had to separate labeled deoxyuridine phosphates from thymidine phosphates. dUTP was eluted in HPLC immediately in front of dTTP, but its radioactivity could be distinguished from that of dTTP by its sensitivity to dUTPase. The procedure is illustrated in Fig. 2 that shows chromatograms of cytosolic and mt extracts before and after treatment with dUTPase. The amount of dUTP is the difference between the two values. The treatment did not affect the radioactivity of any other nucleotide peak.

**HPLC of Medium**—To determine the concentration of bases and nucleosides in the growth medium of cells, we precipitated proteins

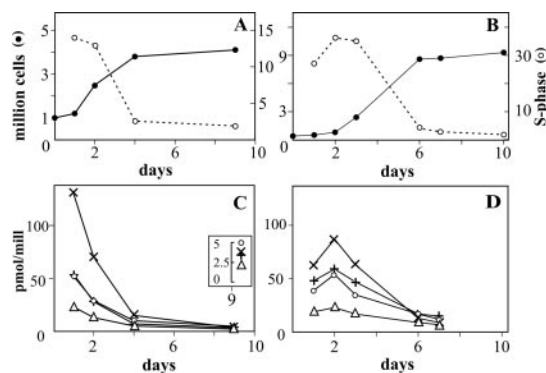


FIG. 3. **dNTP pools in skin and lung fibroblasts during growth.** A–D, we seeded skin (panels A and C) and lung (panels B and D) fibroblasts (400,000 cells) on 10-cm dishes and determined the increase in number and the percentage of cells in S-phase (panels A and B). ●, number of cells; ○, % cells in S-phase. In parallel, we determined the size of each of the four dNTP pools (panels C and D). ×, dTTP; +, dATP; ○, dCTP; ∇, dGTP.

from a portion of the medium with 4 M  $\text{HClO}_4$  (final concentration 0.3 M), neutralized the solution after centrifugation with 4 M KOH, and chromatographed the centrifuged solution on a LUNA C18 column (Phenomex) isocratically with 40 mM ammonium acetate for 20 min followed for 40 min by a linear gradient between 40 mM ammonium acetate and 40 mM ammonium acetate in 30% methanol. Retention times (min) were as follows: uracil, 3.7; thymine, 8.4; deoxyuridine, 12.0; and thymidine, 30. The amount of each nucleoside was determined from its absorption at 260 nm.

**dNTP Pools**—To determine the very small dNTP pools in mitochondria from quiescent cells, we had to modify the previous procedure (25) in the following way. Two portions of different size were incubated at 37  $^{\circ}\text{C}$  for 20 min in a volume of 0.1 ml with 25 pmol of the appropriate template (25), 1 unit of Klenow polymerase, and 0.25  $\mu\text{M}$  [ $^32\text{P}$ ]dATP (2.5  $\mu\text{M}$  in the original procedure) for the assay of dTTP, dGTP, and dCTP. For the assay of dATP, we used 2.5  $\mu\text{M}$  [ $^32\text{P}$ ]dTTP as before. The radioactivity incorporated into the template was determined. The unknown amount of dNTP was calculated from a standard curve. With the exception of the dATP assay, we now used a much lower concentration of dATP and a higher concentration of the polymerase compared with the original procedure. With the new conditions, the results were proportional to the amount of extract used in the assay. For reliable data, it is imperative to use at least two different amounts of extract for each assay. To determine the specific radioactivity of dTTP, we used [ $^3\text{H}$ ]dTTP for the standard curve and determined both [ $^3\text{H}$ ] and [ $^32\text{P}$ ] in both the standard curve and the final assay. The [ $^32\text{P}$ ] values of the standard curve were then used to calculate the amount of dTTP, and the [ $^3\text{H}$ ] values were then used to determine the specific radioactivity.

## RESULTS

**dNTP Pools in Cycling and Quiescent Fibroblast Cultures**—In earlier experiments, we investigated the sources of mt dNTP pools in cultures from established tumor cell lines where between 30 and 40% cells were in S-phase (5, 6). The results defined parameters for mt dNTPs in rapidly cycling cells. Those cell lines had lost contact inhibition, and the cultures could not be obtained in a quiescent state. To achieve this goal, we switched our experiments from tumor cells to two types of human fibroblasts: (i) human skin fibroblasts obtained from three healthy donors (they were used between 5 and 20 passages with similar results) and (ii) a line of lung fibroblasts. We obtained quiescent cells from both types of fibroblasts as described under “Experimental Procedures.”

Fig. 3, panels A and B, show growth curves in 10% serum and the percentage of S-phase cells of skin and lung fibroblasts, respectively. Earlier during growth, 15% skin fibroblasts and 30% lung fibroblasts were in S-phase, whereas after 1 week, the corresponding values were 2 and 3%. Lung fibroblasts grew more rapidly than skin fibroblasts and reached a higher saturation density. The corresponding analyses of the total cellular dNTP pools in Fig. 3, panels C and D, show similar results for

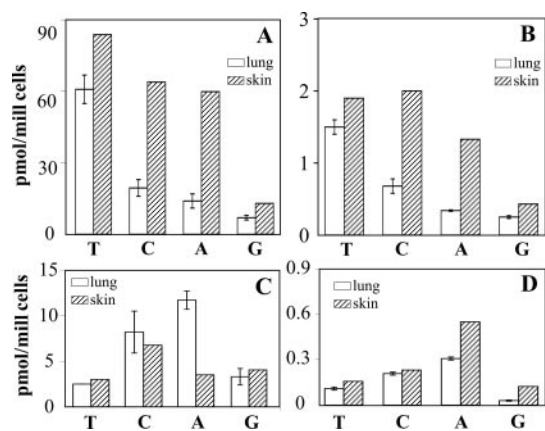


FIG. 4. dNTP pools in cycling (panels A and B) and quiescent cells (panels C and D). We measured dNTP pools in cytosol (panels A and C) and mitochondria (panels B and D) from cycling and quiescent skin and lung fibroblasts. The mean  $\pm$  S.E. show analyses from two separate cultures of lung fibroblasts. T, dTTP; C, dCTP; A, dATP; G, dGTP.

both cell types, both with respect to pool variations during the course of growth and with respect to the relative sizes of the four pools. Thus, all of the pools are much larger during early stages of growth when a larger percentage of the cells is synthesizing DNA with an up to a 50-fold decrease in the size of the dTTP pool and somewhat smaller changes in the other three pools after 1 week. These results agree in general with previous reports (7). During early growth, dTTP is the largest pool, dATP and dCTP both are approximately half its size, and dGTP is the smallest. At the end of the growth curve, when most cells are in the quiescent state, the situation changes and both the dATP and dCTP pools are larger than dTTP. These changes are almost identical for the two cell lines.

**dNTP Pools in Mitochondria from Cycling and Quiescent Cells**—Cells grown for 3 days in 10% serum were taken as cycling cells, whereas contact-inhibited cells kept at least for 1 week in 0.1% serum represented quiescent cells. For both, we analyzed mt and cytosolic pools with results shown in Fig. 4. Both in the cytosol and mitochondria of cycling cells, dTTP was the largest pool and dGTP was the smallest. Only in mitochondria of skin fibroblasts, dCTP equaled dTTP. All four dNTP pools of mitochondria were  $\sim$ 3% cytosolic pools. In quiescent cells a change occurred in the relative pool sizes in both cytosol and mitochondria. dTTP now was smaller than dATP and dCTP.

**Thymidine Kinases and 5'-Deoxynucleotidases in Cycling and Quiescent Fibroblasts**—TK1 activity is generally reported to be low in non-dividing cells, whereas TK2 is not cell cycle-regulated (26). The activity of the two kinases in crude extract can be distinguished from the effects of BVdU. Under the appropriate conditions, BVdU inhibits  $>$ 90% activity of TK2 without affecting TK1. In Table I, we first demonstrate the effect of BVdU on TK activity in crude extracts from one tumor cell line lacking TK1 (OSTTK1<sup>-</sup>) and another cell line containing the enzyme (HOS). In the first case, BVdU completely inhibited thymidine kinase activity but not in the second. The TK1<sup>-</sup> line phosphorylated all of the thymidine by TK2, and the other line used predominantly TK1 with a small contribution from TK2 activity. Cycling lung fibroblasts behave like HOS cells with a dominant TK1, practically not inhibited by BVdU. The total TK activity of cycling skin fibroblasts is less than that of lung fibroblasts, and now we detected the effect of BVdU demonstrating activity of both kinases but with a predominant TK1 component. Turning to quiescent fibroblasts, we noticed the complete inhibition by BVdU in quiescent skin cells, dem-

onstrating the absence of TK1. Lung fibroblasts were also inhibited by BVdU; however, the degree of inhibition depended on the time the cells had spent in 0.1% serum. Inhibition was complete after 34–41 days (1% cells in S-phase), whereas after 7–27 days (2–3% S-phase), 20–30% activity remained, suggesting the presence of TK1 in a small fraction of the cell population. Taken together, the data show that prolonged maintenance of both fibroblast lines in contact-inhibited conditions abolishes TK1 activity but does not impede their TK2 activity.

We also determined the activity of the two deoxynucleotidases cdN and mdN in extracts from cycling and quiescent cells. Determining first the combined activities of cdN and mdN (5 mM dUMP as substrate), we found the same activity in cycling and quiescent cells (Table II). We then used inhibitors to distinguish between cdN and mdN. At 0.2 mM dUMP as substrate, the phosphonate PMcP-U inhibited both enzyme activities, whereas DPB-T strongly inhibited only mdN. Inhibition by PMcP-U confirms the specificity of the assay for a 5'-deoxynucleotidase. The weak inhibition by DPB-T shows that the activity mainly came from cdN. We estimate that mdN represented only 10–15% total activity in both cycling and quiescent cells. The data indicate that the activity of cdN and probably also that of mdN is not cell cycle-regulated.

**Metabolism of dTTP in Quiescent Fibroblasts**—In a first experiment, we labeled skin fibroblasts with 0.1, 0.3, or 1.0  $\mu$ M [<sup>3</sup>H]thymidine for 10, 20, and 60 min. After 20 min, we chased the isotope in some dishes by replacing the radioactive medium with medium containing the same concentration of non-labeled thymidine and continued incubation for 10 or 20 min. At each time point, we measured the appearance of isotope in intracellular thymidine, thymine, and thymidine phosphates (Fig. 5). For dTTP, we also determined the specific radioactivity. The radioactivity of the intracellular thymidine pools (Fig. 5C) attained plateau values already after 10 min, suggesting a rapid equilibration between extracellular and intracellular thymidine. Labeled thymine amounted at all time points to 40–50% thymidine (data not shown). Intracellular pools of both thymidine and thymine increased almost 10-fold when the extracellular thymidine concentration changed from 0.1 to 1.0  $\mu$ M. Thymidine is phosphorylated to dTTP via dTMP and dTDP (cf. Fig. 1). At all of the time points and during the chase, 85–90% total radioactivity was in dTTP, 10–15% was in dTDP, and only 1–3% was in dTMP (data not shown), indicating that the phosphorylation of thymidine to dTMP by TK2 was rate-limiting. The specific radioactivity of dTTP (Fig. 5A) and the total incorporation of radioactivity into dTTP (Fig. 5B) increased with time approaching plateau values after 60 min. The time curves differ from those for thymidine that attained equilibrium already after 10 min, indicating that the phosphorylation by TK2 was a relatively slow process compared with the entrance of thymidine into the cells. With 1  $\mu$ M [<sup>3</sup>H]thymidine in the medium, the final specific activity of the intracellular dTTP approached that of the extracellular thymidine. With 0.1 and 0.3  $\mu$ M [<sup>3</sup>H]thymidine, the plateau values for dTTP were 40 and 60%, respectively, of that of extracellular [<sup>3</sup>H]thymidine. Also the size of the dTTP pool depended on the concentration of thymidine in the medium and increased from  $2.1 \pm 0.2$  (0.1  $\mu$ M thymidine) to  $2.6 \pm 0.4$  (0.3  $\mu$ M thymidine) and  $3.4 \pm 0.5$  (1.0  $\mu$ M thymidine). The difference between 0.1 and 1.0  $\mu$ M thymidine was highly significant ( $p < 0.01$ ). However, note that a 10-fold increase in thymidine concentration results in a  $<$ 2-fold increase in the concentration of dTTP.

In the chase experiments, we were surprised to find a rapid loss of isotope from thymidine phosphates with the concomitant decrease in the specific activity of dTTP (Fig. 5A). In cycling cells, we had found a similar effect of a chase (6) but

TABLE I  
Thymidine kinase activity in cycling and quiescent cells

We determined thymidine kinase activity in crude extracts from cycling and quiescent tumor cells and fibroblasts. Our radioassay with 1  $\mu\text{M}$  [ $^3\text{H}$ ]thymidine measures the sum of TK1 and TK2 activities. To determine TK1 alone, we inhibited TK2 with 50  $\mu\text{M}$  BVdU. Figures in parentheses show the number of assays from different cells. Specific enzyme activity is enzyme milliunits (nmol product/min)-mg protein $^{-1}$ .

	Cycling cells		Quiescent cells	
	TK1 + TK2	TK1	TK1 + TK2	TK1
HOSTK1 $^+$	179	165		
OSTTK1 $^-$	1.0–1.1 (2)	0.03–0.06 (2)		
Lung fibroblasts	205–247 (2)	192–238 (2)	9–12 (3) <sup>a</sup>	1.2–2.8 (3) <sup>a</sup>
Lung fibroblasts			6–8 (2) <sup>b</sup>	0.2–0.5 (2) <sup>b</sup>
Skin fibroblasts	55	43	10–14 (4) <sup>c</sup>	0.3–1.0 <sup>c</sup>

<sup>a</sup> 13–21 days with 0.1% FCS.

<sup>b</sup> 34–41 days with 0.1% FCS.

<sup>c</sup> 7–27 days with 0.1% FCS.

TABLE II  
5'-Deoxynucleotidase activities in cycling and quiescent cells

We determined the activities of the two 5'-deoxynucleotidases, cdN and mdN, in crude extracts from cycling and quiescent lung and skin fibroblasts. Figures in parentheses show the number of assays from different cells. With 5 mM dUMP, we determined the combined activity of the two enzymes. With 0.2 mM dUMP, we used PMcP-U to inhibit both deoxynucleotidases and DPB-T to inhibit only mdN (22) with the remaining activity attributed to cdN. Specific enzyme activity = enzyme milliunits (nmol product/min)-mg protein $^{-1}$ .

Substrate	Inhibitor	Cycling cells		Quiescent cells	
		Skin fibroblasts	Lung fibroblasts	Skin fibroblasts	Lung fibroblasts
5 mM	None	12	14	23	19–25 (3)
0.2 mM	None	1.0–1.4 (2)	1.2	1.3–2.3 (3)	1.9–2.3 (2)
0.2 mM	PMcP-U	0.1–0.4 (3)	0.1	0.2–0.3 (3)	0.3 (2)
0.2 mM	DPB-T	0.8–1.0 (2)	1.0	0.7–1.7 (3)	1.7–2.1 (2)

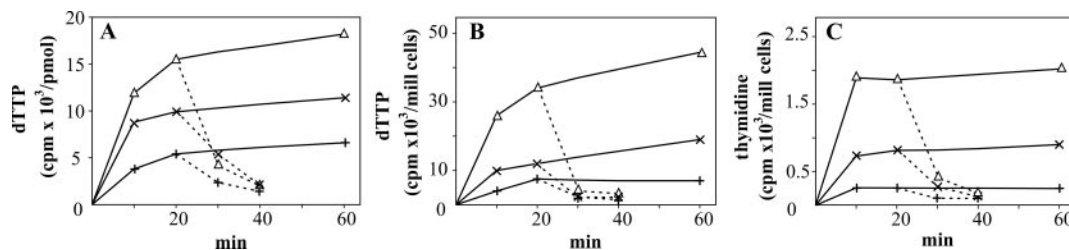


FIG. 5. Time curves for the incorporation of increasing concentrations of [ $^3\text{H}$ ]thymidine into dTTP and thymidine in quiescent cells. We incubated skin fibroblasts maintained for 1 week in 0.1% FCS for the indicated times with 0.1 (+), 0.3 ( $\times$ ), or 1.0 ( $\nabla$ )  $\mu\text{M}$  [ $^3\text{H}$ ]thymidine (20,000 cpm/pmol) and determined in the cell extracts the specific radioactivity of dTTP (panel A), total radioactivity in dTTP (panel B), and total radioactivity in thymidine (panel C). In the chase experiments indicated by the broken lines, we replaced [ $^3\text{H}$ ]thymidine at 20 min with the corresponding amount of non-labeled thymidine and incubated the dishes for an additional 10 or 20 min.

there the turnover of dTTP can be explained by DNA replication that requires a continuous replenishment of dTTP. Quiescent cells do not replicate nuclear DNA, and as elaborated under "Discussion," we must look for a different explanation for the turnover of thymidine phosphates.

The results of Fig. 5 cover the turnover of dTTP in whole cells of quiescent skin fibroblasts. We have carried out several experiments with separated cytosolic and mitochondrial dTTP pools, both with skin and lung fibroblasts, all with similar results. A typical chase experiment concerning the metabolism of 1  $\mu\text{M}$  [ $^3\text{H}$ ]thymidine in cycling and quiescent lung fibroblasts is shown in Fig. 6. Isotope was incorporated into thymidine phosphates within 20 min and then chased for an additional 10 or 20 min. We only show the results concerning the specific activity of the dTTP pool, because measurements of total radioactivity led to the same conclusions. In both cycling (Fig. 6A) and quiescent (Fig. 6B) cells, the radioactivity incorporated into dTTP during the first 20 min of the experiment was rapidly lost during the ensuing chase. Clearly, also mt dTTP is turned over rapidly. A closer comparison of the decay of dTTP in the cytosol and mitochondria shows minor but interesting differences between cycling and quiescent cells. In cycling cells, the two processes occur in parallel. In quiescent cells, the decay

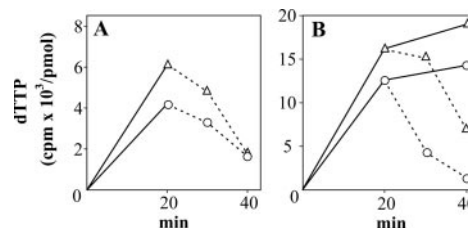


FIG. 6. Comparison of specific radioactivity of dTTP in cytosol and mitochondria from cycling and quiescent lung fibroblasts labeled with [ $^3\text{H}$ ]thymidine. We incubated lung fibroblasts at the third day of growth in 10% FCS (cycling cells, panel A) and after 32 days maintenance in 0.1% FCS (quiescent cells, panel B) with 1  $\mu\text{M}$  [ $^3\text{H}$ ]thymidine (20,000 cpm/pmol) for the indicated times and determined the specific radioactivity of dTTP in the cytosol ( $\nabla$ ) and in mitochondria ( $\circ$ ). In the chase experiment, the labeled medium was replaced after 20 min with fresh medium containing 1  $\mu\text{M}$  cold thymidine and the specific radioactivity of dTTP was determined after an additional 10 and 20 min.

during the first 10 min is more rapid in mitochondria than in the cytosol. This difference may be explained by the fact that, in cycling cells, thymidine is mostly phosphorylated by TK1 in the cytosol, whereas quiescent cells phosphorylate all thymidine by

TABLE III  
*Intracellular pools of thymidine at different concentrations of extracellular thymidine*

We incubated quiescent lung fibroblasts with 0.1–10  $\mu\text{M}$  extracellular [ $^3\text{H}$ ]thymidine and measured at equilibrium the total amount of radioactivity of the intracellular thymidine in cytosol and in mitochondria. From these values and from the specific radioactivity of the [ $^3\text{H}$ ]thymidine in the medium, we calculated the amount of intracellular thymidine, assuming it had the same specific radioactivity as the extracellular deoxynucleoside. The results are compiled from several independent experiments and expressed as pmol/million cells. Figures in parentheses show the number of independent experiments.

Extracellular thymidine ( $\mu\text{M}$ )		0.1	0.3	1.0	10
Intracellular thymidine (pmol/million cells)					
Cytosol	Cycling	ND <sup>a</sup>	0.33	ND <sup>a</sup>	3.5
	Quiescent	0.065	0.29	ND <sup>a</sup>	1.4–2.3 (2)
Mitochondria	Cycling	0.010	ND <sup>a</sup>	ND <sup>a</sup>	0.36
	Quiescent	0.008	0.010	0.042	0.14–0.29 (3)

<sup>a</sup> ND, not determined.

TK2 in mitochondria. An additional difference concerns the values for the specific radioactivity. In quiescent cells, the plateau values for both cytosolic and mitochondrial dTTP approach the specific radioactivity of the external thymidine. Cycling cells attain a plateau value already at 20 min (not shown in this experiment), but the value amounts to only 30% that of the external thymidine. In this case, *de novo* synthesis of dTTP from non-labeled ribonucleotides (*cf.* Fig. 1) dilutes the radioactivity coming from the phosphorylation of labeled thymidine.

The distribution of isotope among dTTP, dTDP, and dTMP in quiescent fibroblasts differed consistently between the cytosol and mitochondria. In five independent experiments,  $80 \pm 5\%$  total radioactivity of the cytosol was dTTP, whereas in mitochondria, the corresponding value was only  $55 \pm 7\%$  (data not shown).

*Pool Changes after Incubation of Cells with Thymidine and Deoxyuridine*—The cells of MNGIE patients are continuously bathed in a body fluid containing 10–20  $\mu\text{M}$  thymidine and deoxyuridine. Quiescent fibroblasts maintained at 10–40  $\mu\text{M}$  extracellular deoxyribosides might provide a model for MNGIE. Therefore, we made a series of experiments to determine the effects of an increase in the concentration of extracellular deoxynucleosides on intracellular thymidine and dNTP pools. We made first the following experiment to determine to what extent our isolation procedure affects the intracellular content of thymidine. Before separating mitochondrial and cellular dNTPs, we washed cell layers four times with buffered saline. To test a possible loss of thymidine during this procedure, we labeled cells for 60 min with 1 and 10  $\mu\text{M}$  [ $^3\text{H}$ ]thymidine, respectively, washed separate dishes either 3, 4, or 5 times with ice-cold saline, fractionated cells after each washing into the cytosol and mitochondria, and measured by HPLC the radioactivity in thymidine and thymidine phosphates in cytosol and mitochondria. At both concentrations of extracellular thymidine, we found only small changes depending on the number of washings demonstrating that most of thymidine and thymine remained in the cells (data not shown). Cell fractions contained 7–8 times more thymidine (as judged from radioactivity) at 10  $\mu\text{M}$  than at 1  $\mu\text{M}$  thymidine but only 2.5 times more dTTP. Similar to the results in Fig. 5, this finding suggests that the cells during incubation at 37 °C were essentially freely permeable to thymidine and that the increased intracellular concentration of thymidine resulted in an increased dTTP pool; however, this increase was not proportional to the thymidine concentration.

In more extensive experiments, we incubated both quiescent and cycling cells with 0.1–10  $\mu\text{M}$  [ $^3\text{H}$ ]thymidine, washed them four times, and measured the radioactivity of thymidine in mitochondria and in whole cells. We then transformed the radioactivity of thymidine to pmol by assuming that the specific activity of the intracellular deoxynucleoside was identical to that of the extracellular thymidine (Table III). Even though

the data varied considerably, they clearly suggest that thymidine is rapidly transported into cytosol and mitochondria and that its concentration mirrors the extracellular concentration within a 100-fold concentration range. Results from single experiment with cycling cells suggested no major difference from quiescent cells.

To test the effects of extracellular deoxynucleosides on dNTP pools, we maintained quiescent lung fibroblasts for up to 41 days in the presence of 10–40  $\mu\text{M}$  deoxyuridine or 10–40  $\mu\text{M}$  thymidine + deoxyuridine in the medium. We analyzed pools in whole cells 13, 23, 34, and 41 days after administration of the deoxynucleosides. Fig. 7 shows only the average of the results for each dNTP, because we found no clear systematic change in the values between 13 and 41 days. For each dNTP, the *first bar* shows the control value (no deoxynucleoside), the *second bar* shows the cells maintained with only deoxyuridine, and the *third bar* shows cells maintained with thymidine + deoxyuridine. Deoxyuridine by itself had no effect. The combination of thymidine + deoxyuridine increased the dTTP pool 3.5-fold, decreased the dCTP pool to 72%, and did not affect the two purine pools. The increase in the dTTP pool was highly significant ( $p < 0.01$  in Student's *t* test), and the decrease in dCTP was not ( $p = 0.1$ ).

Deoxyuridine in the medium did not affect the size of any dNTP pool, suggesting that the increase in cellular dTTP in the presence of thymidine + deoxyuridine in the medium was caused by thymidine. In a separate experiment, we confirmed that also thymidine alone in quiescent cells increased dTTP without affecting other dNTPs (data not shown).

We then made a more detailed study on the effect of thymidine + deoxyuridine, comparing pools in cycling and quiescent cells as well as in the cytosol and mitochondria (Fig. 8). We present all of the data in Fig. 8 as the increase in the size of each pool caused by the inclusion of deoxyribosides in the medium. In the cytosol from both cycling and quiescent skin fibroblasts, the dTTP pool is increased 2-fold in cycling cells and 3.5-fold in quiescent cells. There is a small (1.5-fold) increase in dGTP only in cycling cells and a still smaller decrease (0.72-fold) of dCTP in quiescent cells. A corresponding comparison of mt dTTP pools shows again a larger (3.7-fold) increase in quiescent than in cycling cells (1.7-fold).

From this and several other experiments not shown here, we conclude that only thymidine, but not deoxyuridine, when present in the medium of cultured fibroblasts expanded the intracellular dTTP pool both in the cytosol and in mitochondria and that the effect was more pronounced in quiescent than in cycling cells. Effects on other dNTP pools were minimal.

*Phosphorylation of Deoxyuridine*—Thymidine kinases phosphorylate not only thymidine but also deoxyuridine; therefore, we can expect that deoxyuridine, when present at high concentration in the medium, is phosphorylated by cells. In the two experiments depicted in Fig. 9, we incubated quiescent lung fibroblasts with [ $^3\text{H}$ ]deoxyuridine and determined the radio-

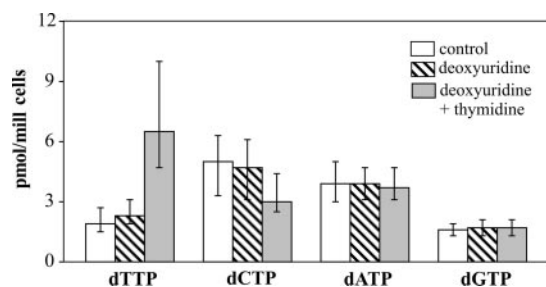


FIG. 7. dNTP pools during long-term incubation of quiescent lung fibroblasts with 10–40  $\mu\text{M}$  deoxynucleosides in medium. We maintained contact-inhibited lung fibroblasts in 0.1% FCS for up to 41 days in culture with either 10–40  $\mu\text{M}$  deoxyuridine or 10–40  $\mu\text{M}$  thymidine + deoxyuridine and removed samples for analyses of dNTP pools of whole cells at 13, 23, 34, and 41 days. The figure shows the averaged values from the four time points with their mean  $\pm$  S.E.

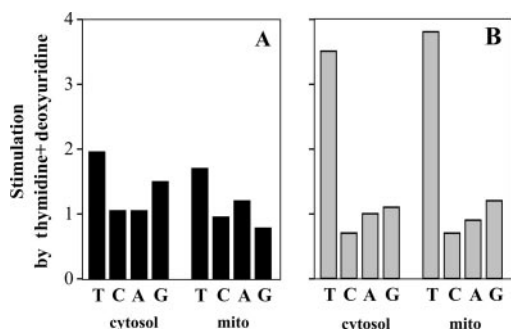


FIG. 8. dNTP pool changes in mitochondria and cytosol of cycling and quiescent cells after incubation with deoxyuridine + thymidine. We cultured lung fibroblasts in the presence of 10–40  $\mu\text{M}$  deoxyuridine + thymidine for 3 days in 10% FCS (cycling cells, panel A) or for 14 days of contact inhibition in 0.1% FCS (quiescent cells, panel B) and measured the size of the dNTP pools in the cytosol and in mitochondria. We also measured dNTPs in cells maintained in parallel without deoxynucleosides. The results are expressed as the ratio between the pool sizes of cells maintained with and without deoxynucleosides. T, dTTP; C, dCTP; A, dATP; G, dGTP.

activity of deoxyuridine phosphates in the separated cytosolic and mt fractions at the indicated time periods.

In the first experiment (Fig. 9A) with 10  $\mu\text{M}$  labeled deoxyuridine, the predominant part of incorporation occurred into dUMP, both in the cytosol and in mitochondria. In the cytosol, but not in mitochondria, we found also a small amount of labeled dUTP amounting to at most 2% of the incorporation into dUMP. If present, we would not have detected such a small amount of dUTP in mitochondria. In the second experiment (Fig. 9, B and C), we used [ $^3\text{H}$ ]deoxyuridine at a 10-fold higher specific radioactivity hoping to be able to detect dUTP also in mitochondria. This experiment involved a chase with non-labeled deoxyuridine. In the cytosol, we found roughly the same amount of dUMP and dUTP as in the first experiment but we now also detected a small amount of dUTP in mitochondria. Treatment of the sample with dUTPase removed the radioactivity from the chromatographic peak in the position of dUTP, and we are confident that it indeed represents dUTP. During the chase, both dUMP and dUTP disappeared rapidly. dUTP was completely gone after 20 min, reflecting its rapid dephosphorylation by intracellular dUTPase.

#### DISCUSSION

Genetic mtDNA depletion syndromes challenge our understanding of the metabolism of mt dNTPs. They can arise from deficiencies in enzymes of dNTP metabolism operating either inside or outside mitochondria. Our model in Fig. 1 suggests the existence of two independent pathways for mt dNTP synthesis. It also postulates a rapid equilibration of nucleotides

between the cytosol and the mt matrix. Why does a deficiency in an enzyme from one pathway (TK2 or dGK) cause disease when there is a second pathway? Why does the deficiency of an enzyme (thymidine phosphorylase) operating in the cytosol cause the depletion of DNA inside mitochondria? We found the two independent pathways in cycling tumor cells containing 30–40% S-phase cells (6). The salvage pathway is required only in quiescent cells, and we propose that the mtDNA depletion syndromes are diseases of quiescent cells.

To substantiate this hypothesis, we determined in this work first the size of the mt dNTP pools in quiescent cells. All four dNTP pools were, as expected, much smaller than in cycling fibroblasts. In cycling cells, the dTTP pool was largest, whereas dATP and dCTP were the largest pools in quiescent cells, both in the cytosol and in mitochondria. The mitochondria of both quiescent and cycling fibroblasts contained 3–4% of each dNTP, similar to earlier results from cycling tumor cells (5). As discussed then, this calculation does not consider total deoxynucleotides because the distribution among monophosphate, diphosphate, and triphosphate differs between cytosol and mitochondria, as we also have found now. However, this does not detract from the general conclusion that each dNTP is quite evenly distributed between cytosol and mitochondria.

Our isotope experiments with labeled thymidine serve to illustrate the similarities and differences in metabolism of thymidine phosphates between quiescent and cycling cells. Both types of cells rapidly equilibrate intracellular thymidine in cytosol and mitochondria with [ $^3\text{H}$ ]thymidine in the medium. From hereon, different paths lead to the synthesis of dTTP. In cycling cells, with predominant TK1 activity, thymidine is salvaged mainly by TK1 in the cytosol and thymidine phosphates are imported into mitochondria (cf. Fig. 1). Equilibrium is reached already after 20 min. However, the largest part of dTTP is produced by *de novo* synthesis and the specific radioactivity of dTTP at equilibrium is considerably lower than that of thymidine supplied from the medium. Quiescent fibroblasts lack TK1 activity and have very low, if any, *de novo* synthesis. TK2 phosphorylates [ $^3\text{H}$ ]thymidine inside mitochondria, thymidine phosphates are exported to the cytosol, and the specific radioactivity of dTTP at equilibrium approaches that of the supplied [ $^3\text{H}$ ]thymidine. The time period to reach equilibrium is considerably longer despite the smaller dTTP pool (2 versus 100 pmol in cycling cells), demonstrating that thymidine phosphorylation by TK2 proceeds much slower than by TK1. The distribution of isotope among monophosphate, diphosphate, and triphosphate of thymidine shows clearly that thymidine kinase is rate-limiting for the formation of dTTP. In both the cytosol and mitochondria, thymidine phosphates are continuously degraded and resynthesized even though in quiescent cells DNA replication does not drain off the dTTP pool. This turnover suggests instead a dynamic equilibrium with thymidine at the level of the dTMP/thymidine substrate cycles (cf. Fig. 1). The two 5'-deoxynucleotidases participating in the cycles are active also in quiescent cells (Table II), and the cycles may be particularly important in such cells for the regulation of the dTTP pool when the allosteric inhibition of ribonucleotide reductase and of TK1 by dTTP is irrelevant.

From the experiments discussed so far, we can draw the following conclusions about quiescent cells. (i) They have very small but measurable dNTP pools both in the cytosol and in mitochondria with the mitochondrial pools reflecting the size of the cytosolic pools. (ii) They rapidly equilibrate internal and external thymidine. (iii) They lack *de novo* synthesis of dTTP and an active TK1 in the cytosol. (iv) They phosphorylate thymidine with TK2 in mitochondria and export thymidine phosphates to the cytosol. (v) Thymidine phosphates, both in

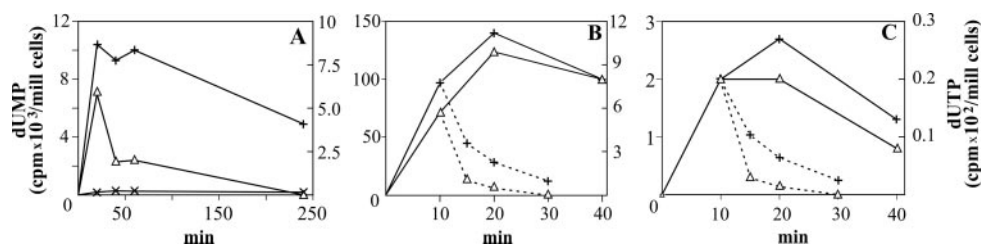


FIG. 9. Phosphorylation of [ $^3\text{H}$ ]deoxyuridine by quiescent lung fibroblasts. In two separate experiments, we added  $15\ \mu\text{M}$  deoxyuridine + thymidine to quiescent lung fibroblasts and incubated them for 24 h. We then changed the medium, and in the first experiment (panel A), we incubated the cells with  $10\ \mu\text{M}$  [ $^3\text{H}$ ]deoxyuridine ( $2000\ \text{cpm}/\text{pmol}$ ) +  $10\ \mu\text{M}$  non-labeled thymidine +  $10\ \mu\text{M}$  uracil (to dilute labeled uracil formed by phosphorylation of deoxyuridine) for up to 240 min, separated cytosolic and mt dNTPs, and determined the amount of labeled dUMP and dUTP. In the second experiment (panel B, cytosol; panel C, mitochondria), we labeled cells with a 10-fold higher specific activity by incubation with  $3\ \mu\text{M}$  [ $^3\text{H}$ ]deoxyuridine ( $20,000\ \text{cpm}/\text{pmol}$ ) + thymidine + uracil and included a chase experiment. For each panel, the ordinate on the left gives counts/min in dUMP and the ordinate on the right gives counts/min for dUTP. Note that the data are not corrected for differences in the specific activity of labeled deoxyuridine in the two experiments. Panel A, +, dUMP in cytosol;  $\times$ , dUMP in mitochondria;  $\nabla$ , dUTP in cytosol. Panels B and C, +, dUMP;  $\nabla$ , dUTP.

the cytosol and mitochondria, undergo a rapid turnover via dTMP/thymidine substrate cycles. These properties agree with the concept that mtDNA depletion syndromes are diseases of quiescent cells. Thus a deficiency of TK2 limits the amount of dTTP for mtDNA replication only in the absence of both ribonucleotide reductase and TK1 and a similar argument can be made regarding a deficiency of dGK.

In the case of MNGIE, the loss of cytosolic thymidine phosphorylase leads to a huge increase in the concentrations of thymidine and deoxyuridine in the body fluids? How does this affect dNTPs? Already in 1973, we found that concentrations of thymidine of  $1\ \text{mM}$  in the medium of cultured cells not only increased their dTTP pool but also the dGTP and dATP pools and greatly decreased the dCTP pool with inhibition of DNA replication (27). We could explain the pool changes from the effect of dTTP on ribonucleotide reduction and demonstrate that the inhibition of DNA replication was caused by the deficiency of dCTP. More recently, HeLa cells growing at  $50\ \mu\text{M}$  thymidine gave much smaller but similar effects (28). After 8 months of growth at high thymidine, their mtDNA showed multiple deletions.

The above discussed pool changes reflected the presence of an active ribonucleotide reductase in cycling cells. Thymidine ( $1\text{--}10\ \text{mM}$ ) has been used to block the cell cycle at the  $G_1/S$  border (29). The effects of increased thymidine concentration on quiescent cells are, however, not known. We wished to mimic the conditions in MNGIE and investigated how quiescent fibroblasts in culture handle a prolonged incubation with  $10\text{--}40\ \mu\text{M}$  extracellular deoxynucleosides. This is a moderate concentration compared with that of thymidine used for cell synchronization but exceeds the amount of thymidine in dialyzed medium at least 100-fold. The intracellular concentration of thymidine (and presumably deoxyuridine) closely followed the extracellular concentration. However, the dTTP pool increased only at most 4-fold both in the cytosol and in mitochondria and remained expanded up to 41 days (Fig. 8). The 4-fold increase is modest when compared with the  $\sim 100$ -fold expansion of the thymidine pool, suggesting a tight control of the size of the dTTP pool, possibly by the dTMP/thymidine substrate cycles. In cycling cells, the relative (but not the absolute) expansion was still smaller. In the cells, both ribonucleotide reductase and TK1 are under strict control from the allosteric inhibition by dTTP. Other dNTP pools were barely affected. dGTP may be slightly increased in S-phase cells, and dCTP may be slightly decreased both in cycling and quiescent cells. The latter result may depend on increased competition between thymidine and deoxycytidine for phosphorylation by TK2.

Deoxyuridine in the medium of quiescent cells did not affect the four dNTP pools (Fig. 7). The cells rapidly phosphorylated deoxyuridine and accumulated in both cytosol and mitochon-

dria a large dUMP pool and a very small dUTP pool, corresponding to 2% of dUMP (Fig. 9). This is the opposite relation to thymidine phosphates where dTMP was 2% dTTP. Making the reasonable assumption that the labeled deoxyuridine phosphates had the same specific radioactivity as the precursor [ $^3\text{H}$ ]deoxyuridine, we can calculate that, in the presence of deoxyuridine,  $10^6$  cells contained 5–7 pmol dUMP and 0.1 pmol dUTP in the cytosol and 0.15 pmol dUMP and 0.001 pmol dUTP in the mitochondria. Thus the cells contained three times more dUMP than dTTP. Similar to thymidine phosphates, both dUMP and dUTP turned over rapidly (Fig. 9).

To what extent do the present results contribute to an understanding of MNGIE? The effects of thymidine and deoxyuridine were small, both the pool changes induced by thymidine and the amount of dUTP formed from deoxyuridine. It is surprising that such minor events should give rise to profound disturbances of mtDNA synthesis. However, in preliminary experiments to be analyzed further, we found a depletion of the mtDNA of quiescent fibroblasts maintained in the presence of  $10\text{--}40\ \mu\text{M}$  thymidine + deoxyuridine for several weeks.<sup>2</sup> Therefore, we must consider how the pool data are related to the development of mtDNA depletion.

The small decrease of the dCTP pool by thymidine, both in cycling and in quiescent cells, can hardly by itself cause DNA depletion. The increase in the size of the dTTP pool provides a more likely explanation. It creates a pool imbalance by shifting the "normal" size relations between dTTP and the other three pools in quiescent cells toward those found in cycling cells, albeit with very different absolute pool sizes. Larger biases than observed here are known to be mutagenic for nuclear DNA replication (30, 31). In MNGIE, the target is mtDNA. In the presence of added thymidine, the increase of the dTTP pool is larger in quiescent than in cycling cells and it is not impossible that it affects mtDNA replication in cells devoid of nuclear DNA replication. It would be interesting to know to what extent *in vitro* mtDNA replication (38) is affected by pool asymmetries.

Also, the incorporation of uracil from dUTP into mtDNA should be considered (17) Uracil incorporation depends on the intracellular dUTP/dTTP ratio. Once incorporated, uracil is excised by uracil-DNA glycosylase (32), creating the prerequisite for DNA strand breaks. In normal cells, dUTP cannot be detected because of its rapid degradation to dUMP by powerful dUTPases in the nucleus and in mitochondria (33). Antifolates that inhibit the synthesis of dTMP from dUMP promote the accumulation of dUTP (34) and decrease the dTTP pool with incorporation of uracil into DNA and cell death (35). In yeast,

<sup>2</sup> G. Pontarin, P. Ferraro, M. L. Valentino, M. Hirano, P. Reichard, and V. Bianchi, unpublished data.

genetic manipulations of dUTPase and uracil-DNA glycosylase have shown the critical importance of these two enzymes for this process (36). The amount of dUTP formed in our experiments resulted in a dUTP/dTTP ratio of ~0.01 in mitochondria, and the resulting incorporation into DNA may be considered too low to result in DNA damage. However, also the very small dUTP pools in humans suffering from folic acid deficiency increase the incorporation of uracil into DNA and were postulated to provoke damage (37). It is also possible that the activities of dUTPase and/or uracil DNA glycosylase differ between fibroblasts and cells from tissues afflicted by MNGIE, resulting in these cells in larger dUTP pools and more extensive DNA damage.

In conclusion, we present here a model for studies of MNGIE. We propose that MNGIE is a disease of quiescent cells and demonstrate the effects of thymidine and/or deoxyuridine at concentrations present in MNGIE on mt dNTP content and metabolism. In our model system, thymidine causes a moderate pool bias with at most a 4-fold increase in dTTP that may interfere with normal DNA replication. It is possible that, in MNGIE cells lacking thymidine phosphorylase, the increase is larger. Deoxyuridine leads to accumulation of small amounts of dUTP resulting in increased incorporation of uracil. Both of these aspects may contribute to the mtDNA depletion found in MNGIE.

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