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 μM 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 μM extracellular thymidine, intracellular thymidine rapidly approaches the extracellular concentration. We note the conditions of MNGIE by maintaining quiescent fibroblasts in 10–40 μM 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.

Mitochondrial Deoxynucleotide Pools in Quiescent Fibroblasts

A possible model for mitochondrial neurogastrointestinal encephalomyopathy (MNGIE)*

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Mitochondrial 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 μM 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 μM extracellular thymidine, intracellular thymidine rapidly approaches the extracellular concentration. We note the conditions of MNGIE by maintaining quiescent fibroblasts in 10–40 μM 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) DNA. Nuclear DNA replication occurs only during S-phase, once during 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% of 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 [3H]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 diphasate) 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-
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 cycle directing its activity in the catabolic direction. This system provides the cell with an intricate mechanism to regulate

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, 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.
Mitochondrial dNTP Pools and MNGIE

From a portion of the medium with 4 mM HClO₄ (final concentration 0.3 mM), neutralized the solution after centrifugation with 4 mM KOH, and chromatographed the centrifuged solution on a LUNA C18 column (Phenomenex) 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 °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 μM [α-32P]dATP (2.5 μM in the original procedure) for the assay of dTTP, dGTP, and dCTP. For the assay of dATP, we used 2.5 μM [α-32P]dATP 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 [1H]dTTTP for the standard curve and determined both [3H] and [32P] in both the standard curve and the final assay. The [32P] values of the standard curve were then used to calculate the amount of dTTP, and the [3H] 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
TK1 activity is generally reported as 90% activity of TK2, with only 3% cytosolic pools. In quiescent cells, contact-inhibited cells kept at least for 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 representative samples. Mitochondria and cytosolic pools were 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 ~5% 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 both the enzymes is synthesized 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.

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

### Table I

<table>
<thead>
<tr>
<th></th>
<th>Cycling cells</th>
<th>Quiescent cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK1 + TK2</td>
<td>179</td>
<td>165</td>
</tr>
<tr>
<td>TK1</td>
<td>1.0–1.1 (2)</td>
<td>0.03–0.06 (2)</td>
</tr>
<tr>
<td>OSTTK1</td>
<td>0.03–0.06 (2)</td>
<td></td>
</tr>
<tr>
<td>Lung fibroblasts</td>
<td>205–247 (2)</td>
<td>192–238 (2)</td>
</tr>
<tr>
<td>Lung fibroblasts</td>
<td>55</td>
<td>43</td>
</tr>
<tr>
<td>Skin fibroblasts</td>
<td>0.3–1.0 (2)</td>
<td></td>
</tr>
</tbody>
</table>

*a* 12–21 days with 0.1% FCS.

*b* 34–41 days with 0.1% FCS.

*c* 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 PMcP-U, 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).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>Cycling cells</th>
<th>Quiescent cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM</td>
<td>None</td>
<td>12</td>
<td>23</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>None</td>
<td>1.0–1.4 (2)</td>
<td>1.3–2.3 (3)</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>PMcP-U</td>
<td>0.1–0.4 (3)</td>
<td>0.2–3.3 (3)</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>DPB-T</td>
<td>0.8–1.0 (2)</td>
<td>0.7–1.7 (3)</td>
</tr>
</tbody>
</table>

### Fig. 5

Time curves for the incorporation of increasing concentrations of [3H]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 (20,000 cpm/pmol) and determined in the cell extracts the specific radioactivity of dTTP.

### Fig. 6

Comparison of specific radioactivity of dTTP in cytosol and mitochondria from cycling and quiescent lung fibroblasts labeled with [3H]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 μM [3H]thymidine (20,000 cpm/pmol) for the indicated times and determined the specific radioactivity of dTTP in the cytosol (\(\Delta\)) and in mitochondria (\(\square\)).

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 experiment concerning the metabolism of 1 μM [3H]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 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
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 the cytosol and mitochondria is shown in Fig. 8. The intracellular thymidine concentration was not a linear function of the extracellular thymidine concentration. The radioactivity of thymidine to pmol by assuming that the specific radioactivity of the thymidine in the medium, is phosphorylated by cells. In the two experiments depicted in Fig. 9, we incubated quiescent lung fibroblasts with 0.1–10 μM extracellular [3H]thymidine and measured at equilibrium the total amount of radioactivity of the intracellular thymidine in cytosol and mitochondria. From these values and from the specific radioactivity of the [3H]thymidine in the medium, we calculated the amount of intracellular thymidine, assuming it had the same specific radioactivity as the extracellular deoxyribonucleoside. The results are compiled from several independent experiments and expressed as pmol/million cells. Figures in parentheses show the number of independent experiments.

<table>
<thead>
<tr>
<th>Extracellular thymidine (μM)</th>
<th>0.1</th>
<th>0.3</th>
<th>1.0</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycling</td>
<td>ND*</td>
<td>0.33</td>
<td>ND*</td>
<td>3.5</td>
</tr>
<tr>
<td>Quiescent</td>
<td>0.065</td>
<td>0.29</td>
<td>ND*</td>
<td>1.4–2.3 (2)</td>
</tr>
<tr>
<td>Cycling</td>
<td>0.010</td>
<td>ND*</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>Quiescent</td>
<td>0.008</td>
<td>0.010</td>
<td>0.042</td>
<td>0.14–0.29 (3)</td>
</tr>
</tbody>
</table>

* ND, not determined.

TK2 in mitochondria.
activity of deoxyuridine phosphates in the separated cytosolic and mt fractions at the indicated time periods.

In the first experiment (Fig. 9A) with 10 μM 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 [5-3H]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 [3H]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 [3H]thymidine inside mitochondria, thymidine phosphates are exported to the cytosol, and the specific radioactivity of dTTP at equilibrium approaches that of the supplied [3H]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 dTTP pools 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

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**Fig. 7.** dNTP pools during long-term incubation of quiescent lung fibroblasts with 10–40 μM deoxynucleosides in medium. We maintained contact-inhibited lung fibroblasts in 0.1% FCS for up to 41 days in culture with either 10–40 μM deoxyuridine or 10–40 μM 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 ± 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 μM 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.
In two separate experiments, we added 15 μM 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 μM [3H]deoxyuridine (2000 cpm/pmol) + 10 μM non-labeled thymidine + 10 μM 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 μM [3H]deoxyuridine (20,000 cpm/pmol) + thymidine + uracil and included a chase experiment. For each panel, the ordinate on the left gives counts/min for 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; ×, dUMP in mitochondria; ◀, dUTP in cytosol. Panels B and C, +, dUMP; ◀, dUTP.

FIG. 9. Phosphorylation of [3H]deoxyuridine by quiescent lung fibroblasts. In two separate experiments, we added 15 μM 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 μM [3H]deoxyuridine (2000 cpm/pmol) + 10 μM non-labeled thymidine + 10 μM 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.

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The cytosol and mitochondria, undergo a rapid turnover via dTMP/thymidine substrate cycles. These properties agree with the concept that mtDNA deletion 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 phosphorolase 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 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% confluence had the same specific radioactivity as the precursor [3H]deoxyuridine, we can calculate that, in the presence of deoxyuridine, 106 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 10–40 μM thymidine + deoxyuridine for several weeks. 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 mitochondria 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,
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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.

REFERENCES